U.PORTO

DOUTORAMENTO

BIOTECNOLOGIA MOLECULAR E CELULAR APLICADA ÀS CIÊNCIAS DA SAÚDE

# Modulation of the Inflammatory Response to Biomaterials: Macrophages and NLRP3 Inflammasome

Daniela Pereira de Vasconcelos

**D**

**2019**

U.PORTO

**Daniela Pereira de Vasconcelos.** Modulation of the Inflammatory Response to Biomaterials:Macrophages and NLRP3 Inflammasome

**D**.ICBAS **2019**

**Modulation of the Inflammatory Response to Biomaterials: Macrophages and NLRP3 Inflammasome**

Daniela Pereira de Vasconcelos

SEDE ADMINISTRATIVA

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

FACULDADE DE FARMÁCIA

DANIELA PEREIRA DE VASCONCELOS

# MODULATION OF THE INFLAMMATORY RESPONSE TO BIOMATERIALS: MACROPHAGES AND NLRP3 INFLAMMASOME

Tese de Candidatura ao grau de Doutor em Biotecnologia Molecular e Celular Aplicada às Ciências da Saúde, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

**Orientador** – Doutora Maria Judite Tavares Moreira Novais Barbosa

**Categoria** – Professor Auxiliar (ICBAS) e investigadora do Grupo Microenvironments for Newtherapies (I3S/INEB).

**Afiliação** – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto (ICBAS); Instituto de Investigação e Inovação em Saúde/Instituto de Engenharia Biomédica, Universidade do Porto (I3S/INEB).

**Coorientador** – Doutora Maria de La Salette de Freitas Fernandes Hipólito Reis Dias Rodrigues

**Categoria** – Professora Associada (FFUP) e líder do grupo de investigação NanoPlatforms for Life (Requimte laboratório associado para a Química Verde).

**Afiliação** – Faculdade de Farmácia, Universidade do Porto (FFUP), Rede de Química e REQUIMTE


3S
INSTITUTO DE INVESTIGAÇÃO
E INOVAÇÃO EM SAÚDE
UNIVERSIDADE DO PORTO


INEB
Instituto de Engenharia Biomédica


requimte
LABORATÓRIO ASSOCIADO
PARA A QUÍMICA VERDE
U.PORTO
FACULDADE DE FARMÁCIA
UNIVERSIDADE DO PORTO


IMIB
Instituto Murciano de
Investigación Biosanitaria
Arrixaca
Hospital Clínico Universitario
"Virgen de la Arrixaca"

***Ao Ricardo, à minha família e amigos,***

# FUNDING

The work included in this thesis was financially supported by:

- FCT PhD Programmes and by Programa Operacional Potencial Humano (POCH), BiotechHealth Programme (Doctoral Programme on Cellular and Molecular Biotechnology Applied to Health Sciences - reference PD/00016/2012), and through the PhD studentship PD/BD/114011/2015.

- FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalization (POCI), Portugal 2020.

- Portuguese funds through FCT - Fundação para a Ciência e a Tecnologia in the framework of the projects: PTDC/SAU-BMA/113030/2009; "Institute for Research and Innovation in Health Sciences" (POCI-01-0145-FEDER-007274); NORTE-01-0145-FEDER-000012, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).

- Pablo Pelegrín was supported by the European Research Council (ERC-2013-CoG 614578), EU COST Program (BM1406), Sysmex funding, Ministerio de Economia, Industria y Competitividad–Fondo Europeo de Desarrollo Regional (project no. SAF2017-88276-R).

# PUBLICATIONS | AWARDS

Under the terms of nº 2, alínea a) do artigo 31º do Decreto-Lei n.º 230/2009, the work presented in this doctoral thesis, is already published or in preparation for publication:

**Article I**

*Review article published*

**Vasconcelos, D.P.,** Águas, A.P., Barbosa, M.A., Pelegrín, P., Barbosa, J.N. (2019). The Inflammasome in Host Response to Biomaterials: Bridging Inflammation and Tissue Regeneration. Acta Biomaterialia, 83, 1-12, 2019. DOI: 10.1016/j.actbio.2018.09.056.
(Parts included in general introduction).
URL: https://www.sciencedirect.com/science/article/pii/S1742706118305853

**Article II**

*Original research article published*

**Vasconcelos, D.P.,** Torre-Minguela, C., Gomez, A. I., Águas, A. P., Barbosa, M. A., Pelegrín P., Barbosa J.N. (2019). 3D Chitosan scaffolds impair NLRP3 inflammasome response in macrophages. Acta Biomaterialia, 2019. DOI 10.1016/j.actbio.2019.04.035.
URL: https://www.sciencedirect.com/science/article/pii/S1742706119302788?via%3Dihub

**Article III**

*Original research article published*

**Vasconcelos, D.P.,** Costa, M., Amaral, I.F., Barbosa, M.A., Águas, A.P., Barbosa, J.N. (2015). Development of an immunomodulatory biomaterial: Using resolvin D1 to modulate inflammation. Biomaterials, 53, 566-573, 2015. DOI: 0.1016/j.biomaterials.2015.02.120.
URL: https://www.sciencedirect.com/science/article/pii/S0142961215002586

**Article IV**

*Original research article published*

**Vasconcelos, D.P.,** Costa, M., Neves, N., Teixeira, J.H., Vasconcelos, D.M., Santos, S.G., Águas, A.P., Barbosa, M.A., Barbosa, J.N. (2018). Chitosan porous 3D scaffolds embedded with resolvin D1 to improve in vivo bone healing. J Biomed Mater Res A, 106 (6), 1626-1633, 2018. DOI: 10.1002/jbm.a.36370.
URL: https://onlinelibrary.wiley.com/doi/full/10.1002/jbm.a.36370

***Article V***

*Original research article in preparation*

**Vasconcelos, D.P.**, Barbosa, M.A. Águas, A.P., Reis, S., Nunes, C., Barbosa, J.N. (2019). Solid lipid nanostructures as a novel delivery system for Resolvin D1: improvement of Resolvin D1 half-life.

***Complementary publications in the field:***

1. Torres A.L., Bidarra S.J., **Vasconcelos D.P.**, Barbosa J.N., Silva E.A., Nascimento D.S., Barrias C.C. (2019). Microtissue engineering for therapeutic vascularization: dynamic endothelial changes upon priming in bioengineered 3D microniches translate into higher vasculogenic/angiogenic potential. *Submitted.*

1. Lourenço A.H., Torres A.L., **Vasconcelos D.P**., Machado-Ribeiro C., Barbosa J.N., Barbosa A.M., Barrias C.C., Ribeiro C.C (2019). Osteoimmunomodulatory properties of strontium-rich injectable hybrid scaffold for bone repair. Mater Sci Eng C.

2. Almeida C.R., Caires H.R., **Vasconcelos, D.P.,** Barbosa, M.A. (2016). NAP-2 secreted by human NK cells can stimulate mesenchymal stem/stromal cell recruitment. Stem Cell Reports, 6:4, 466-473, 2016. DOI: 10.1016/j.stemcr.2016.02.012
URL: https://www.sciencedirect.com/science/article/pii/S2213671116000631?via%3Dihub

3. **Vasconcelos, D.P.,** Costa, M., Amaral, I.F., Barbosa, M.A., Águas, A.P., Barbosa, J.N. (2015). Modulation of the inflammatory response to chitosan through M2 macrophage polarization using pro-resolution mediators. Biomaterials, 37, 116-123, 2015.
DOI: 0.1016/j.biomaterials.2014.10.035
URL: https://www.sciencedirect.com/science/article/pii/S0142961214010825?via%3Dihub

4. **Vasconcelos, D.P.,** Costa, M., Amaral, I.F., Barbosa, M.A., Águas, A.P., Barbosa, J.N. (2013). Macrophage polarization following chitosan implantation. Biomaterials, 34, 9952-9959, 2013. DOI: 10.1016/j.biomaterials.2013.09.012
URL: https://www.sciencedirect.com/science/article/pii/S0142961213010958?via%3Dihub

5. Almeida, C.R*., **Vasconcelos, D.P***., Gonçalves, R.M., Barbosa, M.A. (2012). Enhanced mesenchymal stromal cell recruitment via natural killer cells by incorporation of inflammatory signals in biomaterials. J R Soc Interface, 9 (67): 261–271, 2011
DOI: 10.1098/rsif.2011.0357
URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3243397/pdf/rsif20110357.pdf
*These authors contributed equally to the study.

## Awards

**European Science Foundation (ESF) grant** that covered full conference fee and travel expenses in recognition of outstanding work with the title “Modulation of the inflammatory response to chitosan through M2 macrophage polarization using pro-resolution mediators”, selected for an oral communication during ESF-EMBO conference on Interaction Between the Immune System and Nanomaterials: Safety and Medical Exploitation (4-9 October 2015, Pultusk, Poland).

**Best poster presentation award** – 1st Symposium on Immunomodulation in Cancer and Regeneration: INEB, 2015

# ACKNOWLEDGEMENTS

Ao terminar esta tese de doutoramento, sinto-me absolutamente privilegiada e agradecida pelas escolhas que fiz. Chego ao final com mais perguntas do que as que iniciei este processo. E isso deixa-me muito feliz. Foram 4 anos muito especiais, com altos e baixos como em todos os projetos, mas sem dúvida que os altos foram em maior número. Tive a sorte de ter tido a oportunidade de desenvolver o meu projeto de doutoramento num instituto de excelência onde impera a amizade e a entreajuda. Este é o momento de agradecer a todas as pessoas que contribuíram para que este dia fosse possível e felizmente, tenho muito a quem agradecer por tanto que me deram ao longo destes 4 anos.

As minhas primeiras palavras são para a minha orientadora, **Judite Barbosa**, não há palavras para agradecer tudo o que fez por mim ao longo destes anos. Obrigada por me ter dado asas para voar, sem nunca me ter tirado o chão. Obrigada por me ter dado liberdade para ir fazendo as minhas escolhas e tomando decisões ao longo deste percurso. Graças à Judite chego ao final deste doutoramento completamente autónoma. Como a Judite sempre fez questão de defender e mencionar, um aluno de doutoramento deve ter sentido crítico, deve ser capaz de pensar pela própria cabeça e no final deve ser ele a fazer determinadas opções. Judite, obrigada pela oportunidade de trabalhar e aprender consigo. Obrigada por acreditar em mim, obrigada por toda a ajuda na escrita dos artigos, desta tese e pela disponibilidade em ver/rever tudo o mais rápido possível. Principalmente, obrigada pela amizade e carinho ao longo destes anos. Espero que sinta orgulho em mim e no trabalho que desenvolvemos.

Ao **Professor Mário Barbosa**, pela oportunidade de voltar ao INEB e desenvolver o meu trabalho de doutoramento na sua equipa, o meu muito obrigada. Como o Professor Mário me disse na sessão de posters do Winter Science Club de 2012, “O bom filho à casa torna”. Não podia estar mais certa de que fiz a melhor opção, quando voltei para o INEB. Obrigada por tudo.

Um agradecimento especial ao **Professor Artur Águas**, por me receber sempre tão bem e por ter sempre uma palavra de incentivo e carinho para comigo e para com o meu trabalho. Obrigada pelo entusiasmo ao longo de todo este processo e principalmente obrigada pelo cuidado que sempre demonstrou nas revisões dos artigos. Por último, obrigada por não usar o TrackChanges, fazendo de todas as revisões um exercício deveras interessante.

Agradeço à **Professora Salette Reis**, por ter aceitado ser minha coorientadora, apesar de não me conhecer. Confiou na minha ideia e aceitou o meu pedido de ajuda.

Obrigada por me ter acolhido tão bem no seu laboratório, por ter sempre uma palavra de carinho para comigo e principalmente por ter colocado a Cláudia Pinho no meu caminho.

Obrigada à **Cláudia Pinho** toda a ajuda no desenvolvimento da última parte deste trabalho, pelo entusiasmo e pela disponibilidade ao longo de todo este processo.

À **Madalena**, um obrigada muito especial por toda a ajuda ao longo destes anos. Obrigada pela companhia e pelas conversas durante as horas que passamos em pé a operar ratinhos no biotério. Obrigada por estares sempre disponível para me ajudar, obrigada pela simpatia e carinho com que me recebes sempre. Obrigada por todos os cafés que me deste (devo estar a dever-te dezenas de cápsulas) e pelas bolachinhas antes de entrarmos para o biotério. Por fim, muito obrigada pela amizade ☺.

Agradeço ao **BiotecHealth** e à **Fundação para a ciência e Tecnologia** pelo financiamento deste projeto.

Aos meus colegas da edição de **2015** do **BiotechHealth**, **José P**, **Daniela F**, **Joana M** e **Ana Sara**, agradeço o companheirismo, e a entreajuda ao longo destes 4 anos. **Flávia Castro** e **Joana Marques**, a vocês em especial, obrigada pelos momentos partilhados e pela amizade.

Aos **INEB**ianos, **APF**, **Cristina Ribeiro**, **Meriem**, **Pedro Granja**, **Professor Fernando Jorge**, **Perpétua**, **Ricardo Vidal**, **Dalila**, **Manela**, **Sílvia Bidarra**, **Maria José Oliveira**, **Maria Lazaro**, **Juliana**, **Isabel Amaral**, **Cristina Martins**, **Virgínia**, **Dulce**, **Daniela Azevedo** e a todos aqueles que de alguma forma me ajudaram ao longo destes anos, muito obrigada. **Inês Gonçalves** obrigada por me teres levado para as escolinhas e ensinado o quanto é importante sabermos comunicar o nosso trabalho aos mais pequenos. Foi/é uma experiência única, ver o brilho no olhar dos miúdos sempre que aprendem algo novo com as “cientistas” ☺.

A todos os elementos do grupo **MINT** (atuais e antigos) **Daniel V.** (qual é a probabilidade de na mesma equipa existir um n=2 de alunos de doutoramento com o mesmo nome e o mesmo sentimento em relação ao segundo nome próprio?:)); **Morena**, **Mafalda G**, **João B**; **Cláudia M**; **Pipa L**; **Carla C**; **Susana S**; **Xu**; **Raquel G**; **Inês A, Ana Luísa** e **Nuno Neves**, pelo bom ambiente no laboratório, pelas brincadeiras em momentos mais complicados e por estarem sempre disponíveis para ajudar, o meu muito obrigada. **Grace**, obrigada pela amizade, pela partilha, por seres a pessoa mais pequenina, responsável, trabalhadora e querida do laboratório, e por chegares sempre cedo para ires tomar café comigo☺. Tenho saudades tuas. Um agradecimento especial à **Andreia S**, por todas as discussões cientificas que me ajudaram a aprender e a melhorar o trabalho, por estar sempre disponível para ajudar (mesmo que isso implicasse ter de ficar até tarde no laboratório para terminar o trabalho dela), por cuidar dos problemas do laboratório e mais importante por ser uma excelente pessoa e amiga. Fazes muita falta aos MINTs.

**Zé**, tu mereces um agradecimento especial, foste/és das melhores pessoas que o INEB me deu oportunidade de conhecer. Além de seres um amigo muito especial, és dos melhores colegas de trabalho que se pode pedir. Obrigada por todas as horas que perdeste a ver as minhas coisas, a treinar comigo as apresentações, a tranquilizar-me antes das mesmas pois sou a pessoa mais nervosa à face da Terra e a apoiar-me em todos os momentos. Obrigada por seres juntamente com a **Caty**, a melhor pessoa para partilhar a secretária e formar o 3' perfeito ☺. Especialmente, obrigada por seres meu AMIGO e por todos os momentos bons que partilhámos e ainda iremos partilhar.

O **INEB** é um local de amizades e afetos. Aqui cresci e conheci pessoas que passaram a ocupar um lugar muito especial na minha vida. **ALT**, **Parreira**, **Bianca** e **Rúben**, obrigada pela amizade, pelas conversas, pelos momentos partilhados, por estarem sempre dispostos a ajudar-me e principalmente obrigada por partilharem comigo a mesma CAPETICE. **Aninhas S**, obrigada por seres a menina mais doce e com os melhores abraços de todo o INEB. **Dani Sousa**, muito obrigada pela amizade e por me ajudares na revisão da tese. Gosto muito de todos vocês.

My stay abroad at **Pablo's Pelegrin** Group, was very important for my development as a researcher. To Pablo, my special thanks for welcomed me so well in his group, for all the help with my work and for being an inspiration.

To **Carlos T**, **Ana I** and **Fatima** thank you for your help and everything you have teach me in Murcia, and for your friendship.

To **Ana T**, **Mari Carmen** and **Maria**, thanks for all your kindness and friendship during my stay in Múrcia.

À **Inês Silva**, "a portuguesa" que chegou ao grupo do Pablo, 2 dias depois de mim, e que foi a minha companhia durante 3 meses no autocarro para o Instituto, nos fins de tarde no laboratório, no ginásio, no chocolate quente semanal no CaféLab ☺ e mais importante, durante os fins de semana, o meu muito obrigada. Os últimos meses em Múrcia sem ti foram bastante mais difíceis.

**Estrelinha** e **Inesita**, foi o INEB que nos "apresentou", vocês chegaram e ficaram na minha vida, passando a ser família. Obrigada por estarem sempre presentes, nos bons e maus momentos. Obrigada por me chamarem à razão quando é preciso, obrigada pelo apoio incondicional. Principalmente, obrigada por ficarem genuinamente felizes com as minhas pequenas conquistas, como se fossem vossas. **Star**, obrigada por estares sempre disponível para me ajudares e ouvires, obrigada por tudo o que fazes por mim. Às duas, muito, muito obrigada por fazerem parte da minha vida. Adoro-vos♥.

Um obrigada do tamanho do mundo à minha querida **Caty** ☺. Quantas pessoas terão o privilégio de puderem trabalhar lado a lado com a melhor amiga? ☺...Sou, sem

dúvida nenhuma uma privilegiada e uma sortuda. **Caty**, não há palavras para agradecer tudo o que fizeste por mim ao longo destes anos. Sem dúvida que és uma das responsáveis por esta "viagem" ter sido tão agradável. Obrigada por seres a melhor amiga do mundo, obrigada por tomares as minhas dores e conquistas como se fossem tuas, obrigada por seres das melhores pessoas da minha vida, obrigada por todos os momentos que vivemos dentro e fora do laboratório. Tenho a certeza de que iremos viver muitos mais e muito melhores ☺. Obrigada por tornares os meus aniversários super especiais ao decorares a minha secretária com bandeirolas e post its coloridos ☺.... Começo o dia a chorar de alegria de tão emocionada e feliz que fico com o teu gesto. Obrigada por seres tão especial, diferente e a alegria de quem te rodeia. Obrigada por seres uma inspiração incrível a todos os níveis. És mais que amiga, és uma irmã para mim. Adoro-te muito Cat♥.

**Aninhas,** obrigada pelos bons momentos ao longo de anos e anos de amizade, obrigada pelo apoio e carinho em todo este processo. Obrigada pela surpresa no aeroporto depois do meu aniversário com aqueles balões gigantes, foi o meu primeiro aniversário fora do país, longe do Ricardo, dos meus amigos e família e tu fizeste com que o meu coração transbordasse de felicidade. Não há palavras para te agradecer. Agora que a tese está terminada, a ver se conseguimos estar mais tempo juntas ☺.

**Vasquinho, Jon, Nascimento e Vaninha,** sou a última do grupo a terminar o doutoramento ☺**.** Obrigada por todos os anos de amizade e por de todas as vezes que estamos juntos, mesmo que poucas, pareça que o tempo não passou e estamos novamente na FCUP.

**João Vinagre**, **Nuno Vitorino**, **Nunex** e **Valter** os meus "cunhados do coração", obrigada pelo carinho, amizade, apoio e bons momentos em "Família". A ti **Vinagre**, por resolveres todos os meus problemas informáticos, por teres sempre a resposta certa para qualquer dúvida, por seres um bom amigo e um investigador por quem tenho imenso respeito e admiração, o meu muito obrigada ☺.

Aos meus sobrinhos emprestados, **Manelinho**, **Rodrigo**, **Maria**, **MM**, **MC**, **Flor,** obrigada por serem tão fofinhos e por me deixarem estrafegar-vos de mimos (mesmo que não vos dê muita escolha) ☺.

**Lisinha**, minha querida, muito obrigada pela tua amizade, pelas nossas conversas e por me teres ido visitar a Múrcia ☺.

**Nina**, **Huguinho** e **Zé Maria**, obrigada pela vossa amizade, pelos bons momentos que temos passado e pelo vosso apoio, agora já tenho tempo para ir jantar convosco ☺.

**Ju**, por compreenderes a minha ausência durante estes anos, por nunca me teres cobrado nada, por me teres apoiado sempre e por não permitires que a distância e o tempo afetassem a nossa amizade, o meu eterno obrigada. Prometo que agora vou passar a estar mais disponível para ti e para ver a tua Flor a crescer ☺..

**Bárbara**, a prima “emprestada” que passou a ser a prima “do coração”. Fui para Biologia porque adorava os documentários da BBC, o meu ídolo era o David Attenborough e o meu sonho era ser como ele. Acabei por ser uma Bióloga de laboratório e sem dúvida que para mim foi a melhor opção, não me podia sentir mais realizada☺... Quando te conheci estavas a entrar para a Faculdade e o curso que escolheste foi precisamente Biologia. Agora, és a Bióloga que eu um dia sonhei ser, destemida, aventureira e que vai para o meio de uma floresta tropical com pouquíssimas condições durante dias, estudar e recolher amostras de espécies raras. Obrigada por seres para mim um exemplo de perseverança, por seres sempre tão entusiasta com o meu trabalho e principalmente, obrigada por ficares genuinamente feliz com as minhas conquistas.

**Manuel António**, **Maria José**, **Marta**, **Fábio** e **Rafa**, obrigada por me receberem sempre tão bem, pelo cuidado e pelo carinho ao longo destes anos. As caminhadas e os jantares convosco são sempre uma experiência incrível.

Aos meus sogros, **D. Clarinda** e **Sr. António**, obrigada por me terem recebido com tanto cuidado e carinho na vossa família. Obrigada pelas nossas conversas e momentos partilhados ao longo destes anos, obrigada pelo vosso apoio e principalmente, obrigada por terem disponibilizado a vossa casa quando mais precisamos. Ao meu cunhado **Pedro**, obrigada pelo carinho, boa disposição e apoio ao longo de todo este processo.

**Tia Cidália** e **Tio Luís**, os meus pais na cidade, muito obrigada por serem os melhores tios do mundo. Não há palavras, para agradecer tudo o que fizeram por mim até hoje. Obrigada por estarem sempre perto e disponíveis para me ajudar, obrigada por gostarem tanto de mim, obrigada por ficarem genuinamente felizes por tudo o que vou conseguindo alcançar, obrigada pelas horas de conversas e partilhas, obrigada por serem tão boas pessoas. E mais importante obrigada por serem o melhor exemplo de uma família feliz, humilde e trabalhadora.

**Joaninha**, minha prima favorita, a minha irmã de uma mãe diferente és o orgulho da família Vasconcelos ☺. Obrigada por seres a prima mais doce, mais responsável maravilhosa do mundo ☺. Obrigada pelos teus abraços maravilhosos, obrigada por vibrares com tudo o que me vai acontecendo como se das tuas próprias conquistas se tratassem. Obrigada por seres um exemplo para mim. E mais importante, obrigada por gostares tanto de mim.

Ao **Dinis**, meu cunhadinho do coração, cresci contigo e gosto de ti como um irmão, obrigada por todas as vezes que me vieste trazer aos domingos de madrugada ao laboratório, esperavas que eu terminasse o que tinha para fazer para depois me levares de volta a tempo dos almoços de família. Obrigada por tudo.

Aos meus irmãos, **Célia** e **Hélder**, das pessoas mais importantes na minha vida, obrigada por serem os melhores irmãos, obrigada por todo o apoio ao longo destes anos,

obrigada por compreenderem as minhas ausências, obrigada por ajudarem e cuidarem dos pais, obrigada por tomarem as minhas dores como se fossem vossas. Acima de tudo, obrigada por serem tão meus amigos. Espero que tenham orgulho em mim. Adoro-vos.

**Maria** e **Francisco**, meus queridos sobrinhos, obrigada por serem o melhor presente que a vida me deu até hoje. A tia promete que agora vai ter mais tempo para estar presente e brincar muito convosco ☺. **Maria**, obrigada pelos teus mini abracinhos, obrigada por mesmo já sendo grandinha, ainda gostares dos abraços e do colo da tua tia/madrinha ☺. Espero que tenhas orgulho na Madrinha e que estes anos de estudo e entrega a um projeto possam ser um bom exemplo para ti. Gosto de vocês daqui até à Lua e desde a Lua até aqui ☺.

**Mãe** e **Pai**, obrigada por todo o apoio e compreensão ao longo deste processo. Obrigada por desejarem e fazerem tudo para eu ter uma vida diferente da vossa. Obrigada por todos os sacrifícios que fizeram para que nunca me faltasse nada para eu estudar e ir atrás dos meus sonhos. Obrigada pelos irmãos mais queridos e companheiros que se pode desejar. Obrigada pelos valores que nos transmitiram e obrigada por gostarem dos três de igual forma, acho que é esse o segredo para terem uns filhos que se dão tão bem. **Pai**, obrigada por sempre me ter exigido que chegasse a horas, independentemente do compromisso ser mais ou menos importante, por ser o meu exemplo de entrega e responsabilidade perante um trabalho/função e principalmente, por ainda hoje mesmo eu tendo 32 anos, me querer encher de abraços e beijinhos sempre que me vê, como se eu ainda fosse uma criança. Apesar de eu reclamar que me aperta e que já sou grandinha para isso, adoro que ainda o faça ☺. Obrigada por se emocionar com todas as minhas conquistas, mesmo que algumas delas tenham sido apenas “um simples cortejo da faculdade”. As minhas amigas sabiam sempre onde estava, pois era o pai mais chorão de todos ☺. **Mãe**, obrigada por fazer sempre tudo o que pode e não pode por nós. **Mãe** e **Pai** espero que tenham muito orgulho em mim e que nunca se arrependam das oportunidades que me deram.

**Ricardo,** my soulmate a ti não há palavras que possam expressar o quanto te estou grata. És o meu porto de abrigo, a pessoa que melhor me conhece e me apoia incondicionalmente. Obrigada por compreenderes as horas até tarde no laboratório e as vezes que tive de ir trabalhar ao fim de semana. Obrigada sobretudo, por todas as vezes que me acompanhaste, quer de madrugada porque tinha de dar analgésicos aos animais de 6 em 6 horas, quer ao fim de semana. Obrigada por todas as vezes que vieste comigo terminar alguma coisa e acabaste sempre a ajudar-me, obrigada por todas as vezes que me ajudaste a tirar esponjas de quitosano das placas ☺. Desculpa as férias que não gozamos em 2018. Obrigada por seres sempre curioso e atento sobre tudo o que te vou contando do meu trabalho. Obrigada por gostares de mim incondicionalmente (apesar de

eu ser provavelmente a pessoa com pior feitio). És a pessoa mais importante da minha vida, e agradeço todos os dias a sorte que tenho em partilhar a minha vida contigo. E mais importante e especial, obrigada pela família que vamos agora começar, 2019 vai ser sem dúvida o ano mais importante das nossas vidas ☺. ***Amo você*** ♥.

Foram sem dúvida 4 anos muito felizes. A todos aqueles que fizeram parte e ajudaram a que esta viagem fosse realizada sem sobressaltos e da qual só guardarei boas memórias, o meu eterno obrigado. Termino esta jornada de coração cheio e com a sensação de dever cumprido ♥.

# INDEX OF CONTENTS

# ABSTRACT

Biomaterial implantation gives rises to an inflammatory response, starting with an acute inflammatory response mediated by innate immune cells, namely macrophages and their mediators. If the inflammatory response is not well resolved, leading to a chronic inflammatory response and material encapsulation, the biological function of the implanted biomaterial is compromised. So, it is fundamental to understand how macrophages will recognize and respond to a specific biomaterial.

For the development of this doctoral dissertation work, we hypothesized that modulating the macrophage response to biomaterials, using an immunomodulatory material that will be able to shift macrophage polarization towards a M2 anti-inflammatory, pro-reparative phenotype we could improve implant tissue integration and tissue repair. So, herein we aimed to: (i) understand the role of NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome in the macrophage response to macroscopic 3D chitosan (Ch) scaffolds with different degrees of acetylation (DA); (ii) develop an immunomodulatory biomaterial using Resolvin D1 (RvD1) incorporated in Ch scaffolds to modulate inflammation through M2 macrophage polarization; (iii) assess in an *in vivo* model of bone tissue injury the ability of the developed material to improve bone healing, using a rat model of femoral bone defect.

To accomplish our aims, we started by investigating how 3D Ch scaffolds affect inflammasome activation. Opposite to what is described, we found that Ch scaffolds *per se* were not able to activate NLRP3 inflammasome neither in mouse or human macrophages. Importantly, Ch scaffolds with different DA promoted a general decrease of interleukin (IL)-1β release, NLRP3 inflammasome oligomers and cell death after NLRP3 inflammasome activation, in both cell types. Finally, we observed that 3D Ch scaffolds impaired the priming step of inflammasome activation, decreasing the pro-IL-1β protein expression in mouse macrophages and NLRP3 protein expression in human macrophages.

The role of macrophages on the outcome of tissue repair, lead us to develop an immunomodulatory biomaterial capable to modulate the inflammatory response through M2 macrophage polarization. To achieve this goal, we incorporated RvD1, an endogenous pro-resolving mediator with essential roles in the resolution of the inflammatory response, in 3D Ch scaffolds. A general decrease of inflammatory cells recruited to the implanted site and also to the implant itself was observed with Ch + RvD1 lyophilized. More notably, the Ch +

RvD1 lyophilized was able to increase the percentage of M2 macrophages and decrease the percentage of M1 macrophages leading to an improvement of M2:M1 ratio that has been pointed as a good prognosis for tissue repair. Importantly, a general decrease of pro-inflammatory cytokines in the inflammatory exudates was also verified with Ch + RvD1 lyophilized, corroborating the phenotypic change observed in macrophages by the analysis of specific cell markers.

Afterwards we verified *in vivo* the ability of the developed material to improve bone repair in a model of bone injury. We observed a slightly increase in new bone formation (BV/TV), a significant increase in bone trabecular thickness (Tb.Th) and collagen (Coll) type I. It is important to mention that an increment of Tb.Th and Coll type I is indicator of bone quality and strength. These outcomes advocated that RvD1 has a positive effect on bone healing.

In order to obtain a sustained release of RVD1 and also to increase its activity through time, solid lipids nanoparticles (SLNs) were used as the delivery system. RvD1-loaded SLNs were successfully developed and tested to assess its capacity to decrease the pro-inflammatory cytokines (IL-6 and tumour necrosis factor (TNF)-$\alpha$) in human macrophages stimulated with lipopolysaccharide (LPS). The results demonstrated that RvD1-loaded SLNs presented an anti-inflammatory effect and are non-toxic to macrophages.

The work developed in the scope of this thesis contributed to further dissect the role of NLRP3 inflammasome in macrophage response to macroscopic materials. Particularly, we showed that 3D Ch scaffolds *per se* were not able to induce NLRP3 inflammasome activation, which is an important aspect in the context of tissue repair. Additionally, we were able to develop an immunomodulatory material, that promote the shift from M1 (pro-inflammatory) macrophage phenotype to a M2 (anti-inflammatory, pro-reparative) macrophage phenotype. Although the material did not enhance substantially bone healing, we were able to improve the formation of new bone of high quality. Consequently, we started to develop a new strategy to increase the half-life and the release time of RvD1. The promising results indicated that RvD1-loaded SLNs could be used to promote bone healing.

Taken together, the results obtained will impact the development of new strategies and new targets to modulate inflammatory response to biomaterials, and thus improve biomaterial/tissue integration and tissue repair.

# RESUMO

O processo de implantação de um biomaterial dá origem a uma resposta inflamatória, que se inicia com uma resposta aguda, mediada por células do sistema imune inato, nomeadamente macrófagos e mediadores inflamatórios produzidos por estas células. Quando a resposta inflamatória persiste, leva a uma resposta crónica e ao encapsulamento do material e consequentemente a sua função biológica é comprometida. É por isso fundamental perceber como é que os macrófagos irão reconhecer e responder a um determinado material.

Deste modo, o trabalho desenvolvido nesta tese de Doutoramento foi sustentado na hipótese de que modulando a resposta dos macrófagos a biomateriais, usando um material imunomodulador, eficiente na alteração da polarização dos macrófagos para um fenótipo do tipo M2, anti-inflamatório e pro-reparativo, poderíamos melhorar a integração dos materiais implantados e ao mesmo tempo favorecer a reparação de tecidos. Neste contexto, os seguintes objetivos foram definidos: (i) investigar o papel do inflamasoma NLRP3 na resposta dos macrófagos a esponjas 3D de quitosano com diferentes graus de acetilação; (ii) desenvolver um material capaz de modelar a resposta imune, incorporando Resolvina D1 (RvD1) nas esponjas de quitosano a fim de obter uma modulação da inflamação através da polarização dos macrófagos para um perfil do tipo M2; (iii) avaliar num modelo animal de lesão óssea, a capacidade do material desenvolvido favorecer a reparação óssea.

Para atingir os objetivos propostos, começou-se por investigar de que forma as esponjas 3D de quitosano influenciam a ativação do inflamasoma NLRP3. Contrariamente ao que está descrito, verificamos que as esponjas de quitosano por si só, não são capazes de ativar o inflamasoma NLRP3 em macrófagos quer de ratinho quer de humanos. É importante destacar que as esponjas de quitosano com diferentes graus de acetilação, promoveram um decréscimo generalizado da interleucina (IL)-1$\beta$; da formação de oligomeros do inflamasoma NLRP3 e morte celular após ativação do NLRP3. Finalmente, observamos que as esponjas de quitosano afetam o primeiro sinal de ativação do inflamasoma, diminuindo a expressão da proteína pro-IL-1$\beta$ em macrófagos de ratinho e a expressão da proteína NLRP3 em macrófagos humanos.

O papel decisivo dos macrófagos na reparação de tecidos, levou-nos a desenvolver um biomaterial imunomodulador capaz de modular a resposta inflamatória através da

polarização dos macrófagos para um fenótipo tipicamente M2. Para atingir este objetivo, incorporamos RvD1, um mediador endógeno com funções cruciais na resolução da resposta inflamatória, em esponjas 3D de quitosano. Foi observada uma diminuição geral de células inflamatórias não só recrutadas para o local do implante, mas também a colonizar o implante de Ch + RvD1 liofilizada. verificou-se ainda que este material foi capaz de aumentar a percentagem de macrófagos M2 e diminuir a percentagem de macrófagos M1 levando a um aumento da relação M2:M1, que é considerado indicador de um bom prognóstico para a reparação de tecidos. É importante notar que uma diminuição geral das citocinas pró-inflamatórias nos exsudatos inflamatórios também foi verificada com o Ch + RvD1 liofilizada, corroborando a alteração fenotípica observada nos macrófagos pela análise de marcadores celulares específicos.

Em seguida, verificamos num modelo *in vivo* de lesão óssea a capacidade do material desenvolvido contribuir para uma melhoria na reparação óssea. Observamos que o material foi capaz de induzir um ligeiro aumento na formação de novo osso (BV / TV), um aumento significativo na espessura trabecular (Tb.Th) e no colagénio tipo I. É importante mencionar que um aumento de Tb.Th e colagénio tipo I é um indicador de qualidade e força do tecido ósseo. Estes resultados demonstram que a RvD1 tem um efeito positivo na reparação óssea.

Para obter uma libertação mais sustentada de RvD1 e também para aumentar a atividade ao longo do tempo, decidimos usar nanopartículas lipídicas sólidas (NLSs) como sistema de entrega de RvD1. NLSs carregadas com RvD1 foram desenvolvidas com sucesso e testadas para avaliar a sua capacidade em diminuir citocinas pró-inflamatórias (IL-6 e TNF-$\alpha$) em macrófagos humanos estimulados com LPS. Verificou-se que as NLSs com RvD1 têm um efeito anti-inflamatório e não são tóxicas.

Globalmente, o trabalho desenvolvido no âmbito desta tese contribuiu para melhorar o conhecimento do papel do inflamasoma NLRP3 na resposta de macrófagos a materiais macroscópicos. Demonstramos que as esponjas 3D de quitosano, por si só, não foram capazes de induzir a ativação do inflamasoma NLRP3, sendo uma característica importante no contexto da reparação de tecidos. Além disso, conseguimos desenvolver um material imunomodulador, que promove a mudança do fenótipo M1 (pró-inflamatório) para um fenótipo M2 (anti-inflamatório, pró-reparativo). Embora o material não tenha melhorado substancialmente a reparação óssea, fomos capazes de induzir a formação de novo osso de elevada qualidade. Consequentemente, começamos a desenvolver uma nova estratégia para aumentar a atividade e o tempo de libertação da RvD1, os resultados

alcançados até ao momento são bastante promissores, indicando que as NLSs carregadas com RvD1 poderão ser usadas para promover a reparação óssea.

Em suma, os resultados obtidos terão impacto no desenvolvimento de novas estratégias para modular a resposta inflamatória a biomateriais, melhorando assim a integração do biomaterial bem como a reparação do tecido em questão.

# List of Abbreviations

| | |
|---|---|
| **μCT** | Microcomputed tomography |
| **ANOVA** | Analysis of variance |
| **APC** | Allophycocyanin |
| **ARLs** | AIM2-like receptors |
| **ASC** | Associated speck like protein containing a caspase recruitment |
| **ATP** | Adenosine triphosphate |
| **BALB/c** | Bagg albino |
| **bCap** | Biomimetic calcium phosphate coating |
| **BLS** | Bone lining cells |
| **BMDM** | Mouse bone marrow derived macrophages |
| **BMPs** | Bone morphogenetic protein |
| **BMU** | Basic multicellular units |
| **BSA** | Bovine serum albumin |
| **BV** | Bone Volume |
| **Caco-2** | Caucasian colon adenocarcinoma |
| **CAPS** | Cryopyrin-associated periodic syndromes |
| **CARD** | C-terminal caspase activation and recruitment domain |
| **CCL2** | C-C motif chemokine ligand 2 |
| **CCL5** | C-C motif chemokine ligand 5 |
| **CCR7** | C-C chemokine receptor type 7 |
| **CD** | Cluster of differentiation |
| **cGAS-STING** | Cyclic GMP-AMP synthase-stimulator of interferon genes |
| **Ch** | Chitosan |
| **CLRs** | C-type lectin receptors |
| **ColI** | Collagen |
| **DA** | Degree of acetylation |
| **DAMPs** | Damage associated molecular patterns |
| **DAPI** | 4',6-Diamidino-2-Phenylindole, Dihydrochloride |
| **DC** | Dendritic cells |
| **DDA** | Degree of deacetylation |
| **DHA** | Docosahexaenoic acid |
| **DLS** | Dynamic light scattering |
| **DNA** | Deoxyribonucleic acid |
| **DPBS** | Dulbecco's phosphate-buffered saline |

| | |
|---|---|
| **ECM** | Extracellular matrix |
| **EDTA** | Ethylenediamine tetraacetic acid |
| **ELISA** | Enzyme-Linked Immunosorbent Assay |
| **ELS** | Electrophoretic light scattering |
| **EOR** | 15-prostaglandin dehydrogenase eicosanoid oxireductase |
| **EPA** | Eicosapentanoic acid |
| **ERK** | Extracellular signal–regulated kinases |
| **FACS** | Fluorescence-activated cell sorting |
| **FBGCs** | Foreign body giant cells |
| **FGF-2** | Fibroblast growth factor |
| **FT-IR** | Fourier transform infrared spectroscopy |
| **GAPDH** | Glyceraldehyde 3-phosphate dehydrogenase |
| **HMGB1** | High mobility group protein B1 |
| **HSP** | Heat shock protein |
| **IκB-α** | NF-kappa-B inhibitor alpha |
| **IFN-γ** | Interferon-gamma |
| **Ig** | Immunoglobulin |
| **IL** | Interleukin |
| **IL-Ra** | Interleukin-1 receptor antagonist |
| **JNK** | c-Jun N-terminal kinases |
| **KO** | Knockout |
| **LDH** | Lactate dehydrogenase |
| **LPS** | Lipopolysaccharide |
| **LRR** | Leucine-rich repeat |
| **LxA4** | Lipoxin A4 |
| **M-CSF** | Macrophage colony-stimulating factor |
| **MAPK** | Mitogen activated protein kinases |
| **MCP-1** | Monocyte chemotactic protein |
| **MIP-1** | Monocyte inflammatory protein |
| **MMPs** | Matrix metalloproteinases |
| **MSCs** | Mesenchymal stromal cells |
| **Mw** | Molecular weight |
| **NATCH** | Nucleotide – binding domain |
| **NF-κB** | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| **NK** | Natural killer cells |
| **NLRP3** | NOD-like receptor containing pyrin domain 3 |

| | |
|---|---|
| **NLRs** | NOD-like receptors |
| **OPG** | Osteoprotegerin |
| **PAMPs** | Pathogen associated molecular patterns |
| **PBS** | Phosphate buffered saline |
| **PDI** | Polydispersity index |
| **PE** | Phycoerythrin |
| **PLG** | Poly(lactide-co-glycolide) |
| **PMMA** | Poly(methyl metacrylate) |
| **PMNs** | Polymorphonuclear leukocytes |
| **PRRs** | Pattern recognition receptors |
| **PUFAs** | Polyunsaturated fatty acids |
| **Pycard** | Apoptosis-associated speck-like protein containing a CARD |
| **PYD** | Pyrin domain |
| **RA** | Rheumatoid arthritis |
| **RANKL** | Receptor activator of NF-κB |
| **RLRs** | RIG-I-like receptors |
| **ROI** | Region of interest |
| **ROS** | Reactive oxygen species |
| **RPM** | Rotations per minute |
| **RT** | Room temperature |
| **RvD1** | Resolvin D1 |
| **SDS** | Sodium dodecyl sulfate |
| **SEM** | Scanning electron microscopy |
| **SiNPs** | Silica nanoparticles |
| **SLE** | Systemic lupus erythematosus |
| **SLNs** | Solid-lipid nanoparticles |
| **SPMs** | Specialized pro-resolving mediators |
| **Tb.Th** | Trabecular thickness |
| **TCPS** | Tissue culture plates |
| **TGF-β1** | Transforming growth factor-β1 |
| **TLRs** | Toll-like receptors |
| **TNF-α** | Tumour necrosis factor-α |
| **TNT** | Titania nanotubes |
| **TV** | Tissue volume |
| **UTP** | Uridine triphosphate |
| **VOI** | Volume of interest |

| | |
|---|---|
| **WT** | Wild-type |
| **ZP** | Zeta Potential |

# CHAPTER I

## GENERAL INTRODUCTION

**This chapter included material from the review article "The inflammasome in host response to biomaterials: bridging inflammation and tissue regeneration". published in Acta Biomaterialia, 83, 1-12, 2019. (doi: 10.1016/j.actbio.2018.09.056)**

# GENERAL INTRODUCTION

## 1. Biomaterials – Concepts and Developments

Biomaterials are used in several different clinical applications, from implants and prosthesis (e.g., hip implants and artificial heart valves) to tissue regeneration and drug delivery. Since the decade of 1960, millions of patients have their quality of life improved through the use of biomaterials or biomedical devices. Biomaterial development has greatly change over time and nowadays biomaterials can be classified according to their evolution in “first”, “second” and “third” generation. The first generation of materials was developed between 1960 and 1970 to be used in the human body. The major goal of the first developed biomaterials was to “achieve a suitable combination of physical properties to match those of the replaced tissue with a minimal toxic response in the host” [1]. The principal feature of these materials was the biological “inertness” with a minimal interaction between the “bioinert” implant and host tissues [1]. In the 1980s Professor Bonfield and his research group have focused their research efforts in the development of a new generation of biomaterials. This work led to an innovative composite material, named as Hapex™, a combination of a polymeric matrix of polyethylene and dispersed bioactive phase of hydroxyapatite (HA) [2]. A second generation of biomaterials had been developed with the purpose of being “bioactive”. These new developed materials would elicit a controlled reaction within the host [3, 4]. After that, different compositions of bioactive glasses, ceramics, glass-ceramic, polymers and composites were developed. The clinical success of “bionert” and bioactive implants has been fundamental to the medical needs with the population ageing. However, between one third to half of the applied prostheses tend to fail within 10-25 years, requiring that patients have a revision surgery [5, 6]. So, the challenge for the twenty-first century, was the development of a third-generation of biomaterials that were able to stimulate specific cellular responses at the molecular level [7, 8]. Third-generation of biomaterials are being designed to activate genes stimulating the tissue repair. Two alternative approaches of repair could be used: (i) *tissue engineering* that uses progenitor cells seeded and differentiated in resorbable scaffolds. Tissue engineered constructs are then implanted in the host to replace damage tissues and (ii) *in situ* tissue regeneration that uses biomaterials in the form of powders, solutions, or microparticles to stimulate local tissue repair through the release at controlled rates of biochemical products, such as growth factors. The release of these biochemical products will activate and stimulate local cells that in turn will produce other factors, stimulating generations of growing cells [7, 8]. The main benefit of both approaches is the genetic control of tissue repair processes [7-9].

Biomaterials are a growing industry and it was reported that the world market is expected to reach 149,17 billion USD per year by 2021 with an expected compound annual growth rate of 16% [10]. In the last decades the definition of biomaterials has been intensely refined. Professor Williams in 1986 defined a biomaterial as "… a substance that has been engineered to take form which, alone or as part of a complex system, is used to direct, by control of interactions with components of living systems, the course of any therapeutic or diagnostic procedure" [11]. More recently a group of world experts in the biomaterials field establish a new definition. A biomaterial is now defined as "a material designed to take a form which can direct, through interactions with living systems, the course of any therapeutic or diagnostic procedure" [12].

Over the last years, we have perceived a change in the concept of an ideal biomaterial. Instead of being a passive material design to diminish host responses, biomaterials are now developed to trigger desired immunological responses and therefore enable its integration and subsequent tissue repair [15]. It is now accepted that a successful biomaterial implantation can be achieved by controlling the activation of the immune system. With this concept a new area of research is emerging, the "Regenerative Immunology". Can the "immunomodulatory biomaterials" became the fourth-generation of biomaterials?

## 2. Inflammatory response to Biomaterials

The inflammatory response to implanted biomaterials has been deeply investigated over the last years. James Anderson has provided us several landmark papers concerning the biological responses to biomaterials [13-15].

The inflammatory response can be defined as an acute response to tissue injury directed at limiting damage to the body, and it is started through the detection of signals of acute damage or changes of the steady state [16]. The process of biomaterial implantation results in damage to tissues or organs of the host. This injury together with changes in the homeostatic mechanisms will lead to an inflammatory response to the implanted biomaterial [14, 17]. A series of events are initiated upon the implantation process, beginning with an acute inflammatory response that in some circumstances may lead to a chronic inflammatory response, a foreign-body reaction, and the deposition of a collagenous fibrous capsule around the implant. The efficacy of biomedical devices can be affected by the extent and duration of the inflammatory process, having a direct impact on biomaterial stability and compatibility [14, 15]. The tissue response to biomaterials is commonly described as a sequence of events that are started by the biomaterial implantation

procedure, additionally to its presence [14, 15]. We will briefly review these responses starting with (i) blood-material interactions; (ii) release of danger signals by injured cells; (iii) acute inflammation; (iv) chronic inflammation; (v) foreign body reaction.

**(i) Blood-material interactions:** The inflammatory response is always initiated due to injury caused in connective tissue. Shortly after injury, changes in vascular flow and permeability occur, followed by the exudation of fluid, proteins and blood cells from the vascular system into the affected tissues [18]. Almost immediately proteins adsorb to the biomaterial surface [19, 20]. This layer of adsorbed proteins (type of proteins, concentration and conformation upon adsorption) will define the initiation of the coagulation cascade, complement system, platelets and immune cells leading to the formation of a transitional fibrin matrix at the implant site (Fig. 1A) [21].

**(ii) Release of danger signals by injured cells:** Following tissue injury, danger signals the ‘‘alarmins” are promptly released by cells undergoing necrosis. Alarmins are the endogenous equivalent of pathogen-associated molecular patterns (PAMPs) including for example heat shock proteins, ATP and uric acid. Alarmins are capable of recruit and activate different immune cells such as macrophages and dendritic cells (DCs), being recognized trough pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), scavenger receptors and purinergic receptors [22-24], and thus promoting inflammation [25, 26]. As a consequence of biomaterial implantation, alarmins will be released by injured cells at the implant site due to the surgical procedure (Fig. 1A) [27].

**(iii) Acute inflammation:** This step of the inflammatory response is of relative short duration and is mainly characterized by the rapid recruitment of polymorphonuclear leukocytes (PMNs) to the implant site through the release of chemoattractants by activated platelets and endothelial cells. The PMNs will then initiate a phagocytic response together with the secretion of proteolytic enzymes and reactive oxygen species (ROS). Due to size disparity, phagocytosis will most likely not occur and the destructive agents released by these cells may corrode the material surface (Fig. 1B). Several chemokines such as monocyte chemotactic protein (MCP-1) and macrophage inflammatory protein (MIP-1$\beta$), that are chemoattractants and activators of monocytes, macrophages, immature DCs and lymphocytes, will be secreted by activated PMNs. Commonly, PMNs will disappear from the implant site in the first two days after biomaterial implantation, [17, 26, 28, 29].

**(iv) Chronic inflammation:** When the inflammatory stimuli persist, a chronic inflammation will progress, being the macrophage one of the central cell type of this phase

of the inflammatory response. Macrophages release a great number of biological active inflammatory mediators such as tumor necrosis factor-a (TNF-$\alpha$), interleukin (IL)-8, IL-1$\beta$, MCP-1 and MIP-1$\beta$ among others (Fig. 1C). Macrophages are considered as having a fundamental role in wound healing and tissue repair since they exhibit extraordinary plasticity and in response to environmental cues can change their physiology, inducing distinct cell populations with different functions. This has originated the division of macrophages into two major extreme phenotypes (M1 and M2). The classically activated, pro-inflammatory, cytotoxic macrophage phenotype, labelled as M1, promotes pathogen killing and is related with classic signs of active inflammation, mostly with chronic inflammation. The alternatively activated, anti-inflammatory macrophage phenotype, labelled as M2, supports immunoregulation tissue repair and remodeling [30-34].

**(v) Foreign body reaction:** Since macrophages can only phagocytose particles up to 5 mm, when the particle size is larger, they will fuse and form foreign body giant cells (FBGCs) (Fig. 1B). It is described in the literature that after fusing in FBGCs, macrophages show a decrease in the phagocytic activity together with an enhanced degradative capacity due to the release of reactive species, thus creating a highly degradative environment at the biomaterial surface [17, 35, 36]. Macrophages and FBGCs can be found at the implanted biomaterial surface for the lifetime of the implant. There is a fibrous encapsulation around the biomaterial due to fibroblast recruiting factors secreted by FBGCs resulting in its activation and collagen (Coll) deposition. This fibrous capsule will impair the implant function because it will be isolated from the local tissue environment [15, 37, 38].

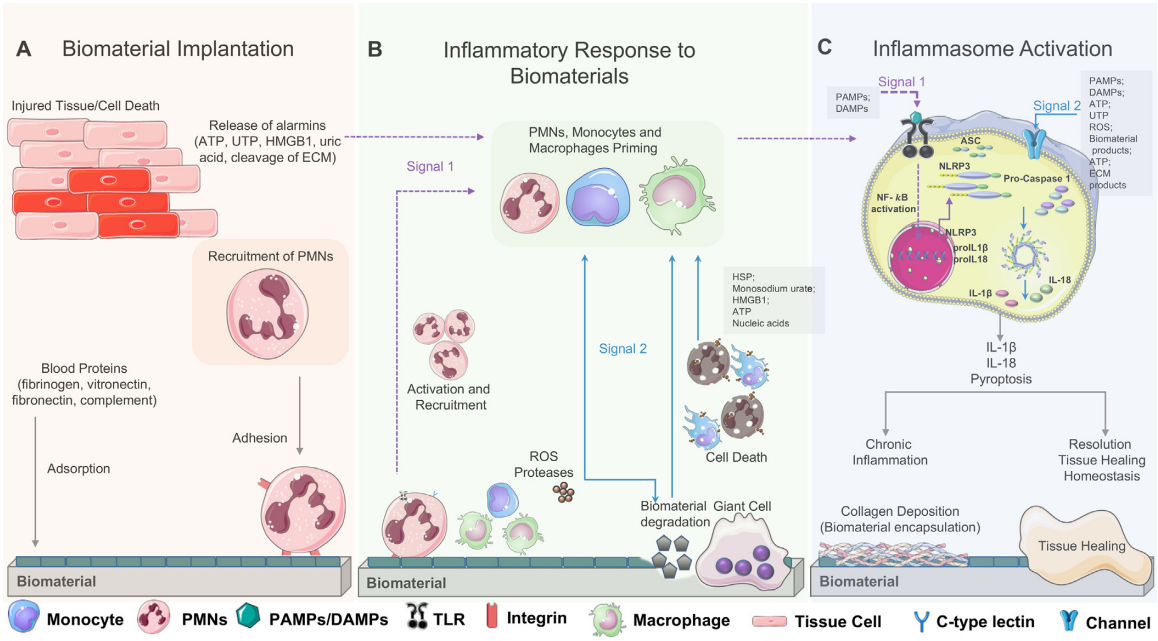



**Figure 1. The inflammasome in the immune response to an implanted biomaterial.** (A) Biomaterial implantation: The process of implantation of a biomaterial causes injury to cells. Danger signals released from injured cells (such as alarmins, High mobility group protein B1 (HMGB1), ATP and UTP) results in the recruitment and activation of PMNs, monocytes and resident macrophages, via PRRs engagement. Well-known damage associated molecular patterns (DAMPs) include ATP, nucleic acids, heat shock protein (HSP), monosodium urate, HMGB1 and inflammatory cytokines. The adsorption of blood proteins to material surface will further recruit immune cells. (B) Acute inflammatory response to biomaterials: Immune cells secrete proteolytic enzymes and ROS that will degrade the biomaterial surface and extracellular matrix (ECM) components. Endogenous danger signals are usually released from stressed or necrotic cells and also damaged ECM during acute inflammation. (C) Inflammasome activation: Activation of NOD-like receptor containing Pyrin Domain 3 (NLRP3) inflammasome, composed of NLRP3, associated speck like protein containing a caspase recruitment domain (ASC), and pro-caspase-1, is regulated by two-step signals: The first signal (signal 1) can be danger signals released from injured tissues and immune cells that will enhance the expression of inflammasome components and target proteins via activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). The second ‘‘activation” signal (signal 2) promotes the assembly of inflammasome components, that involves three major mechanisms, including generation of ROS, lysosomal damage (phagocytosis of biomaterial degradation products), and the potassium efflux. Inflammasome assembly leads to caspase-1 activation that in turn cleaves the pro-forms of cytokines IL-1β and IL-18 as well as gasdermin D that induce the pyroptotic inflammatory cell death. The perpetuation of the inflammatory cascade culminates either in resolution of inflammation, return to homeostasis and tissue healing or in chronic inflammation and biomaterial encapsulation.

## 2.1. Key role of Macrophages

Macrophages display remarkable plasticity, changing their physiology in response to environmental cues [31]. Macrophages are strategically positioned throughout the body

tissues, scavenging debris and dead cells. These cells play a decisive role in tissue repair, secreting cytokines and chemokines that directly impact inflammatory processes and tissue repair [39-41]. Élie Metchnikoff, more than 100 years ago, proposed that the key to immunity is “stimulate the phagocytes” [42]. Since there, immunologists have been occupied in understanding the role of macrophages in host defense. Macrophages mature and are activated in response to environmental stimulus acquiring specialized and distinct functional phenotypes. Similar to Th cell nomenclature system, a division has been proposed for macrophage activation: classic *vs* alternative, M1 and M2, respectively. In response to interferons (IFNs) and TLRs, macrophages undergo M1 (classical) and in response to IL-4/IL-13 signaling undergo M2 (alternative) activation (alternative) [43, 44]. These states correlate to the Th1 – Th2 polarization of T cells [44-46]. The M1 macrophages are characterized by the production of high levels of pro-inflammatory cytokines, reactive nitrogen and oxygen species and strong antimicrobial and tumoricidal activity. On the other hand, the M2 phenotype is considered to be involved in the promotion of tissue remodeling, tumor progression and to have immunoregulatory functions [43].

Tissue repair after injury is a critical biological process that is essential to the survival of all organisms [47]. After tissue injury, due to infection, toxic or mechanical action, an inflammatory response is initiated in response to DAMPs from dead or dying cells or PAMPs from invading organisms [48]. These danger signals induce a complex inflammatory response that is characterized by the recruitment, proliferation, and activation of neutrophils, macrophages, natural killer cells, B cells, T cells, fibroblasts, epithelial cells, endothelial cells, and stem cells, that together orchestrate tissue repair [49]. Once wound-healing is well organized, the inflammatory response resolves rapidly, and normal tissue architecture is restored, but if tissue repair becomes dysregulated, can lead to fibrosis or scaring, that could impair tissue function. Wound-healing is a tightly regulated process and is driven by the interactions between immune cells and other cell types such as fibroblasts and myofibroblasts as well the temporary plasticity of immune cells [50]. The initial cellular response after injury is highly influenced by cytokines, chemokines, MMPs, and other inflammatory mediators, released by tissue resident macrophages. Several studies showed an impairment in tissue repair when macrophages are depleted in an early phase of wound healing [51-53].

Due to their plasticity [31], during wound healing macrophages switch from an inflammatory phenotype, to a tissue repair to a resolving phenotype, having a critical role in the regulation of all phases of repair or fibrosis [54]. M1 macrophages arrive early at the injury site and

are responsible for the recruitment of effector cells and for the initiation of an effective defense to pathogens and the clearance of tissue debris [55]. Chronic M1 macrophages delay tissue repair [56]. In a well balance wound healing, inflammatory macrophages (M1) switch to an anti-inflammatory, homeostatic wound healing macrophages (M2). M2 macrophages guarantee that the tissue-damaging inflammatory response is suppressed, and normal tissue architecture is restored. If the process is not well controlled, persistent inflammation can lead to fibrosis [39, 54, 56].

Macrophages represent a major target in regenerative medicine. Strategies to modulate macrophages suggest the capacity of a better control of wound healing and tissue repair and regeneration. Biomaterials either lager scale or in the form of nanoparticles, soluble factors or even specific cell types could be used to modulate macrophage-mediated repair response.

### 3. Resolution of Inflammation

Inflammation is a crucial step in an efficient host defense. It is a response to pathogen invasion and also to tissue injury. However, to re-establish tissue homeostasis it is necessary to resolve the inflammatory response, in order to prevent increased tissue injury and to minimize the development of chronic inflammation, therefore enabling tissue repair and regeneration [57-59].

The resolution of inflammation and the recovery of homeostasis was for many years considered to be a passive process. It was thought that local inflammatory stimuli would just fade or ‘‘burn out” with time, allowing tissues to drain, repair and return to normal function. It is today accepted that resolution of inflammation is an active process that is highly regulated. Specialized immunoresolvents have been recently described as having a fundamental role, together with anti-inflammatory cytokines as IL-10, in the termination of inflammation triggering pathways that signal the termination of the acute phase of the inflammatory response. These mediators include a group of endogenous molecules: resolvins, lipoxins, protectins and maresins, collectively coined as specialized pro-resolving mediators (SPMs) [60-62].

In the initial phase of inflammation, lipid mediators such as eicosanoids including prostaglandins and leukotrienes, synthesized from membrane-released arachidonic acid of activated cells, have a central role as local mediators in the advance of an inflammatory

condition, inducing an effective chemotactic response of leukocytes whose activation is associated to the local release of pro-inflammatory cytokines. A high increase in the production of inflammatory mediators such as prostaglandins and leukotrienes is associated with an advance from acute to chronic inflammation. In a second phase, an active shift in the type of mediators leads to the production of immunoresolvents initiating the resolution of inflammation. Transcellular metabolism of arachidonic acid by lipoxygenase/lipoxygenase interaction pathways gives rise to SPMs [63, 64]. These endogenous lipid pro-resolution mediators are generated through complex pathways being the (i) lipoxins derived from endogenous fatty acids (arachidonic acid), while (ii) resolvins, (iii) protectins and (iv) maresins are derived from dietary fatty acids, specifically omega-3 fatty-acids [65].

(i) **Lipoxins** (lipoxygenase interaction products) are considered to be effective stop signals for PMNs, limiting their recruitment to sites of inflammation through the reduction of vascular permeability and stimulating the return to homeostasis; they also induce nonphlogistic recruitment of macrophages that are required for wound healing and for the uptake of apoptotic PMNs [66-68].

(ii) **Resolvins** (resolution-phase interaction products) induce several functions in the resolution of inflammation such as the regulation of cytokines and reactive oxygen species; the prevention of PMNs infiltration; the increase phagocytosis of apoptotic PMNs that will clear the lesion and lower the magnitude of the response and thus promote tissue regeneration [66, 69].

(iii) **Protectins** (the term was introduced due to the general anti-inflammatory and protective actions) reduce PMNs recruitment and reinforce the clearance of apoptotic PMNs by macrophages [70].

(iv) **Maresins** (macrophage mediators in resolving inflammation) were described as being produced by macrophages that have homeostatic functions, these mediators also support the removal of apoptotic PMNs by macrophages contributing to the re-establishing of tissue homeostasis [71, 72].

General evidences of resolution are the general decrease of pro-inflammatory cytokines, phagocytosis of apoptotic PMNs and clearance of the inflammatory debris. SPMs stimulate re-epithelialization, wound healing, and tissue regeneration reducing the pro-inflammatory

chemical mediators. Chronic inflammation and fibrosis will occur if inflammatory resolution fails [73].

**4. The new trend in biomaterial development**

For many years, it was accepted that the key for long term durability and function of an implanted biomaterial was its ability to elicit a minimal inflammatory response, since it was considered to be an adverse reaction. Within the last years, this paradigm of the host-biomaterial response has been intensely refined [74]. During several decades biomaterial engineering was dedicated on the development of passive biomaterials to minimize the host response, but it has now been understood that allowing specific biological responses is beneficial for both biomaterial integration and performance [75]. In view of this, biomaterials development has change from ‘‘immune-evasive” to ‘‘immune-interactive” biomaterials to allow the modulation of the inflammatory response improving healing and regeneration [76].

It has now became clear that the immune system is fundamental in orchestrating and defining the nature of the repair response [58, 77], and that without injury and ensuing inflammation, regeneration or repair does not occur [78]. The coordination between inflammation and its resolution is required for successful tissue repair and regeneration [59]. Nowadays, there is growing evidence that the immune response supports repair and provides local tissue protection [79]. The link amongst repair and immune response is complex and both positive and negative roles are described. The result of the tissue healing process can change from incomplete healing and repair that may cause scarring or fibrosis, to complete restoration of the tissue functions being this significantly affected by the immune response [58].

John Hunter, a famous Scottish surgeon, in 1794, wrote that ‘‘inflammation in itself is not to be considered as a disease, but as a salutary operation consequent to some violence or some disease” [80]. Hunter’s rational is rather interesting taking in consideration the recent findings of inflammation being crucial in tissue repair and regeneration.

An impressive number of immune mediators cooperate in every step of the tissue healing process. For example, the macrophage response is crucial for an effective tissue remodeling following biomaterial implantation, since macrophages are rather important in the process of tissue healing [81], and if macrophage infiltration is prevented, healing is

severely impaired [36]. Therefore, modulating the immune system response, namely specific immune cell types, is a valid strategy to support tissue regeneration [82].
Recovery of tissue integrity and return to homeostasis following injury is a central property of all organisms and the immune system is of key importance in defining the quality of the repair process [16, 83]. Recently, new and unexpected roles of immune cells have been described in the promotion of a local environment favorable for effective cell replacement and restoration of tissue integrity. Hence, an in-depth knowledge of the mechanisms controlling the inflammatory response and how it is related with the healing process, will be an important milestone in tissue repair [18, 19].

The challenge nowadays, is to develop capable biomaterial and delivery systems to regulate tissue healing through immune-mediated mechanisms. The next generation of regenerative approaches may progress from typical ‘‘biomaterial-, stem cell-, or growth factor-centric approaches” to an ‘‘immune-centric” approach, following the modulation of the immune system as a way of stimulating repair of tissues and organs [82].

### 4.1. Immunomodulatory biomaterials

Traditionally, the immune system has been viewed by biomedical engineers as an enemy to the adequate design of biomaterials, as a coordinator of the host response that decreases the duration and function of implants. However, interest is increasingly growing on engineering biomaterials to wisely control the immune system by enhancing or suppressing immune reactions [84].

The emergence of tissue engineering and regenerative medicine has motivated the development of novel biomaterials with additional and precise functions, such as the ability to change inflammatory and innate immune response [85].

The conception of biomaterials that are able to modulate the immune system response is a developing field that is evolving together with advances in immunology. There is solid hope on the potential of biomaterials to elicit appropriate immune responses through the modulation of immune cell function, the so called immunomodulatory biomaterials [86].

The use of biomaterials to change immune responses is creating interesting new approaches in different research areas such as cancer immunotherapy, vaccination, establishing tolerance in organ transplantation and treatment of autoimmune disorders [84].

However, in this thesis we will focus on the repair of damage tissues using immune-mediated strategies that is emerging as an innovative approach. Engineering biomaterials to control the immune system may encourage the development of therapies that stimulate pro-regenerative immune responses, leading to an improved tissue repair [87].

These so-called immunomodulatory biomaterials should ideally influence immune cell function promoting tissue healing and the integration of the implant while supporting its function [88]. Different strategies are used in biomaterial-based immunomodulation such as (i) tuning of the chemical properties of biomaterials; (ii) changing the physical properties of the materials (iii) incorporation of bioactive molecules either anti-inflammatory drugs or pro-resolution mediators or growth factors; (iv) biomaterials based on decellularized extracellular matrix (ECM) and (v) cell therapy methods either by including immune cells or by inducing their recruitment [76].

The studies described in the literature attempting to modulate immune responses are mostly focused on the macrophage, namely in macrophage polarization (Fig. 2). This is because macrophages are highly plastic cells [43, 89] that play a decisive role in inflammation and also in the coordination of tissue repair, fibrosis and tissue regeneration [39].

The development of structures that either mimic or use components or decellularized ECM will allow the establishment of a microenvironment favorable for healing and repair [90]. Brown *et al*. [91] have demonstrated that there is an association between early macrophage response to implanted ECM scaffold materials and the result of tissue remodeling probably associated with M1 vs. M2 macrophage response, being increased ratios of M2:M1 macrophages associated with positive remodeling outcomes (Fig. 2). Franz *et al*. [81] have investigated different artificial ECM derivatives and suggest that these materials could be used as coatings for biomaterials allowing the modulation of macrophage functions during the healing response, since they were able to *in vitro* impair the polarization of human M1 macrophages.

The delivery of bioactive molecules such as cytokines or pro-resolution mediators has provided rather interesting results. Gower *et al*. [85] were able to modulate leukocyte infiltration and phenotype after a poly(lactide-co-glycolide) (PLG) scaffold implantation using a gene-therapy approach consisting in the localized delivery of IL-10, decreasing the leukocyte inflammatory response. Spiller *et al*. [92] designed a scaffold that allowed the sequential delivery of interferon-gamma (IFN-$\gamma$) followed by IL-4, in order to promote the transition of M1 to M2 macrophages. Chen *et al*. [93] have used the same rationale and

developed a system of double hydrogel layers on titania nanotubes (TNT) to achieve a controlled release of IL-4 and IFN-γ. We have developed an immunomodulatory strategy based on the local delivery of SPMs, namely lipoxin A4 and resolvin D1, and we were able to *in vivo* shift the macrophage phenotypic profile towards a M2 reparative response [94, 95].

Exploring the physicochemical properties of biomaterials has also led to some promising outcomes. Shayan *et al*. [96] have used nanopatterned bulk metallic glasses to modulate murine macrophage polarization and concluded that nanopatterned surfaces lead to a more constructive tissue repair with higher vascularization and increased M2 to M1 ratio, when compared to flat surfaces. Wang *et al*.[97] have produced macroporous electrospun poly-caprolactone scaffolds with different fiber size and concluded that macrophages cultured on thicker-fiber scaffolds tended to polarize into M2 phenotype, whereas those cultured on thinner-fiber scaffolds expressed mainly M1 phenotype. Lee *et al*. [98] have performed a chemical surface modification in a titanium implants using the divalent cations calcium and strontium and were able to up-regulate M2 macrophage phenotype expression. Li *et al*. [99] have developed titanium implants doped with magnesium with the objective of assessing the macrophage response both *in vitro* and *in vivo* and were able to induce a higher percentage of M2 macrophages and higher concentrations of the anti-inflammatory cytokines IL-4 and IL-10.

The next generation of biomaterials will be developed upon knowledge of the biology of inflammation and healing and will regulate biological responses with precision [75]. It is essential to understand which cells and/or mediators of the immune system can be used to actively stimulate regeneration [100]. Material science has also a great deal to offer to the field of immunology through the design of different biomaterial-based immunomodulatory approaches.

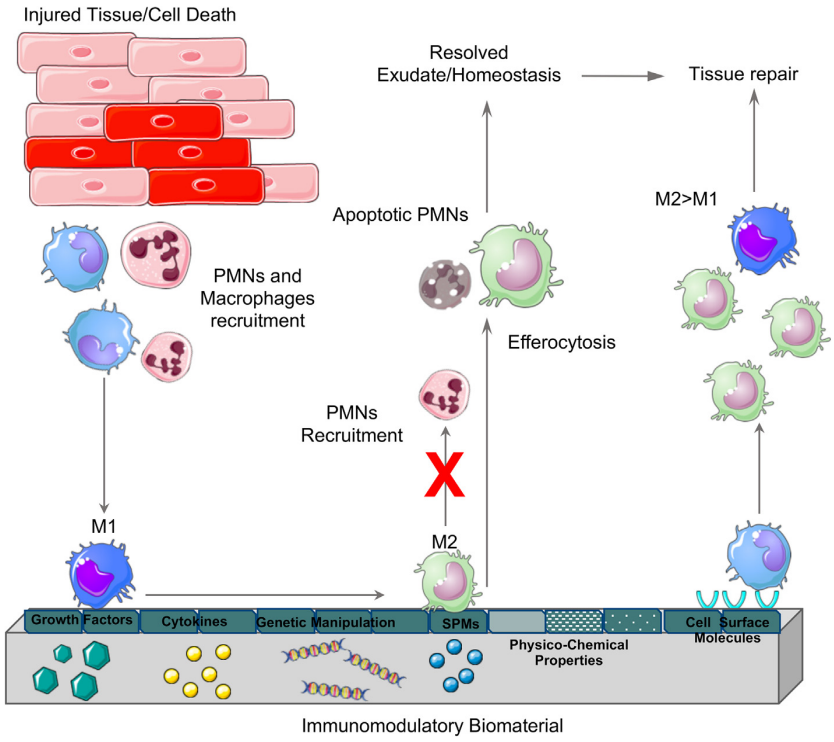



**Figure 2. Modulation of macrophage interaction with biomaterials.** Implantation of a biomaterial triggers an acute inflammatory response, resulting in the recruitment of polymorphonuclear leukocytes (PMNs) and macrophages that will initially polarize to M1 pro-inflammatory macrophages. Afterwards, the immunomodulatory biomaterial will release anti-inflammatory agents (such as cytokines or specialized pro-resolving mediators (SPMs)) leading to changes in the macrophage polarization towards a M2 pro-healing phenotype, promoting implant integration and tissue healing.

## 5. Inflammasomes

### 5.1. What is the inflammasome?

As explained before, inflammation is initiated on the recognition of signs of acute damage or disturbances of the steady state and mainly mediated by the production of soluble factors like cytokines. The innate immune system has several PRRs that upon activation induce the production of different pro-inflammatory cytokines. PRRs can be subdivided into two major classes based in their subcellular location: TLRs and C-type lectin receptors (CLRs) are transmembrane proteins; while the RIG-I-like receptors (RLRs) and the NOD-like receptors (NLRs), reside in the intracellular compartments. These receptors recognize and activate in response, for example, to distinct PAMPs and also host-derived signals produced during tissue damage or homeostasis disturbances called damage-associated molecular patterns (DAMPs) [101, 102].

The concept of inflammasome was introduced in the beginning of this century by Jürg Tschopp. The term inflammasome was coined to describe an intracellular multiprotein complex that perceives pathogenic microorganisms and sterile stressors, being responsible for the activation of the highly pro-inflammatory cytokines IL-1β and IL-18 [103]. Inflammasome is derived from the word inflammation – to reflect the function of this complex - and the suffix ‘‘some” from the Greek ‘‘soma” that means body, which is generally used to define several molecular complexes such as liposome or ribosome [104].

The inflammasome complex (Fig. 3) consists of a cytosolic sensor that could be a PRR of the NLRs or AIM2-like receptors (ARLs) families, in some cases includes an adaptor protein called apoptosis-associated speck like protein containing a caspase recruitment domain (ASC), and an effector protein that in the canonical inflammasomes is caspase-1 [105]. ASC is a bipartite molecule that contains both an N-terminal Pyrin domain (PYD) and a C-terminal caspase activation and recruitment domain (CARD), enabling it to bridge the inflammasome sensor with PYD domain (NLRs or ALRs) and the effector pro-caspase-1. Additionally, to NLRs and ALRs, other proteins with a PYD domain could also assemble ASC to form inflammasomes, such as the protein Pyrin [106].

Different PRRs have been identified to form inflammasomes, including NLRP1, NLRP3, NLRC4 or AIM2, among others. In some conditions the combined activation of two inflammasomes could contribute to the inflammatory response [107-109]. The assembly of these PRRs in pentameric or heptameric structures oligomerize ASC in filaments and these ASC filaments recruit caspase-1 leading to the formation of the inflammasome. The inflammasome complex oligomerize in response to a varied set of inflammation-inducing stimuli including PAMPs and DAMPs and are appreciated as an important sensing system that allows the host to mount an effective immune response [110]. This response is mediated by the activation of caspase-1 within the inflammasome, a response that induces by one hand the cleavage of pro-IL-1β and pro-IL-18 into their mature biological active forms and a special type of cell death termed pyroptosis upon cleavage of gasdermin D. Pyroptosis is executed by the formation of pores in the plasma membrane by the insertion of the resulting N-terminus fragment of gasdermin D and the leakage of intracellular content including mature IL-1β and IL-18 cytokines (Fig. 4).

The NOD like receptor containing pyrin domain 3 (NLRP3) inflammasome (previously known as cryopyrin or NALP3) is presently the most fully characterized inflammasome and consists of the NLRP3 scaffold, the ASC adaptor protein and caspase-1, together with the accessory protein, NIMA – related kinases (NEK7) that maintain NLRP3 oligomer in active

state [111, 112]. The NLRP3 inflammasome is primarily expressed in monocytes, macrophages, granulocytes, dendritic cells, and also in epithelial cells and osteoblast, being its expression in myeloid cells highly inducible [113]. This inflammasome is initially primed in response to diverse signals (Fig. 4, priming ‘‘signal 1”), including *de novo* translation driven by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and post-transcriptional modifications, including phosphorylation, dephosphorylation, deubiquitination, nitrosylation and ribosylation [114]. Upon NLRP3 priming, diverse endogenous host-derived activators (such as extracellular ATP, uric acid crystals or hyaluronan), environmental-derived molecules (such as asbestos and silica particles) and several PAMPs are recognized by myeloid cells and induces the oligomerization of NLRP3 [102, 115, 116]. This second activator signal (Fig. 4, activation for complex formation ‘‘signal 2”) is transduced by various mechanisms such as ROS production or lysosomal damage, being a key common step the intracellular potassium efflux [113, 117, 118]. Therefore, the NLRP3 inflammasome recognize an optimal intracellular milieu [119].

Furthermore, different mutations in NLRP3 results in a protein conformation with increase ability to oligomerize and induce spontaneous activation of the NLRP3 inflammasome in the absence or with a low threshold of activators. These mutations lead to autoinflammatory syndromes in humans known as Cryopyrin-associated periodic syndromes (CAPS), characterized by recurrent inflammatory flares [120].

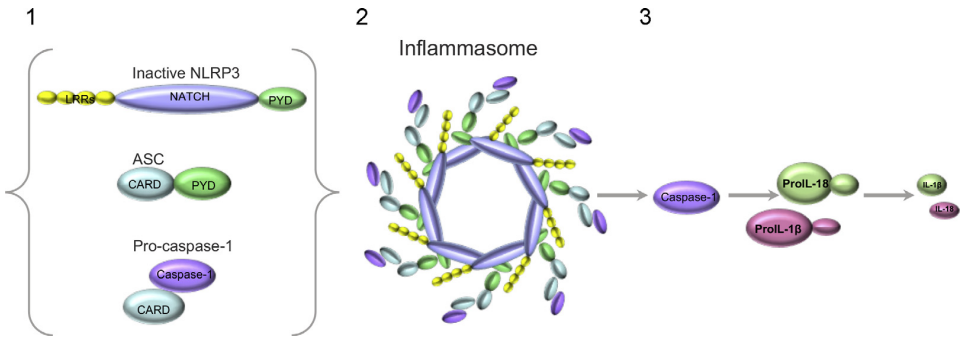



**Figure 3. NLRP3 Inflammasome assembly.** (1) NLRP3 recruits procaspase-1 through ASC to form the inflammasome. (2) Within the inflammasome, procaspase-1 undergoes autocatalytic processing, resulting in active caspase-1 which in turn cleaves the pro-IL-1β and pro-IL-18 into the mature and active form (3). NLR: NOD-like receptor; PYD: pyrin domain; LRR: leucine-rich repeat; NATCH: central nucleotide domain; ASC: apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD). Adapted from [121].

### 5.2. The importance of the inflammasome in the innate immune response

Our knowledge on the molecular mechanisms underlying the different functions of the innate immune system has significantly advanced in the past decade, in particular the activity of the inflammasome in leading innate immune responses, being now considered a central platform for a correct innate immune response [115] and recognized as one of the cornerstones of the intracellular surveillance system [122].

The innate immune system is capable to distinguish non-pathogenic or commensals from pathogenic microbes, but the mechanisms behind this feature is still unknown. Matzinger *et al*. [123], to explain these unanswered questions of the ‘‘self-from-nonself model”, proposed a different hypothesis, ‘‘the danger hypothesis”. This model suggests that an efficient immune response will be triggered through an antigen presentation in the context of a danger signal and not only by the foreignness of the antigen. Interestingly, an increasing number of studies disclose an important function of the inflammasome in the sensing of a controversial signal: danger [104]. The discovery that the NLRP3 inflammasome can be activated by host-derived molecules supports the idea that the innate immune system senses endogenous indicators of cellular danger or stress [115], as in the sterile immune response to allografts [124].

In addition, the effective activity of the inflammasome in guiding innate immune responses is clearly revealed by some heritable and acquired diseases in which the dysregulation of the NLRP3 inflammasome activity due to mutations that affect its structure is observed, and also by the success with which many of these diseases can be treated using IL-1$\beta$ receptors or an antagonist [115, 120].

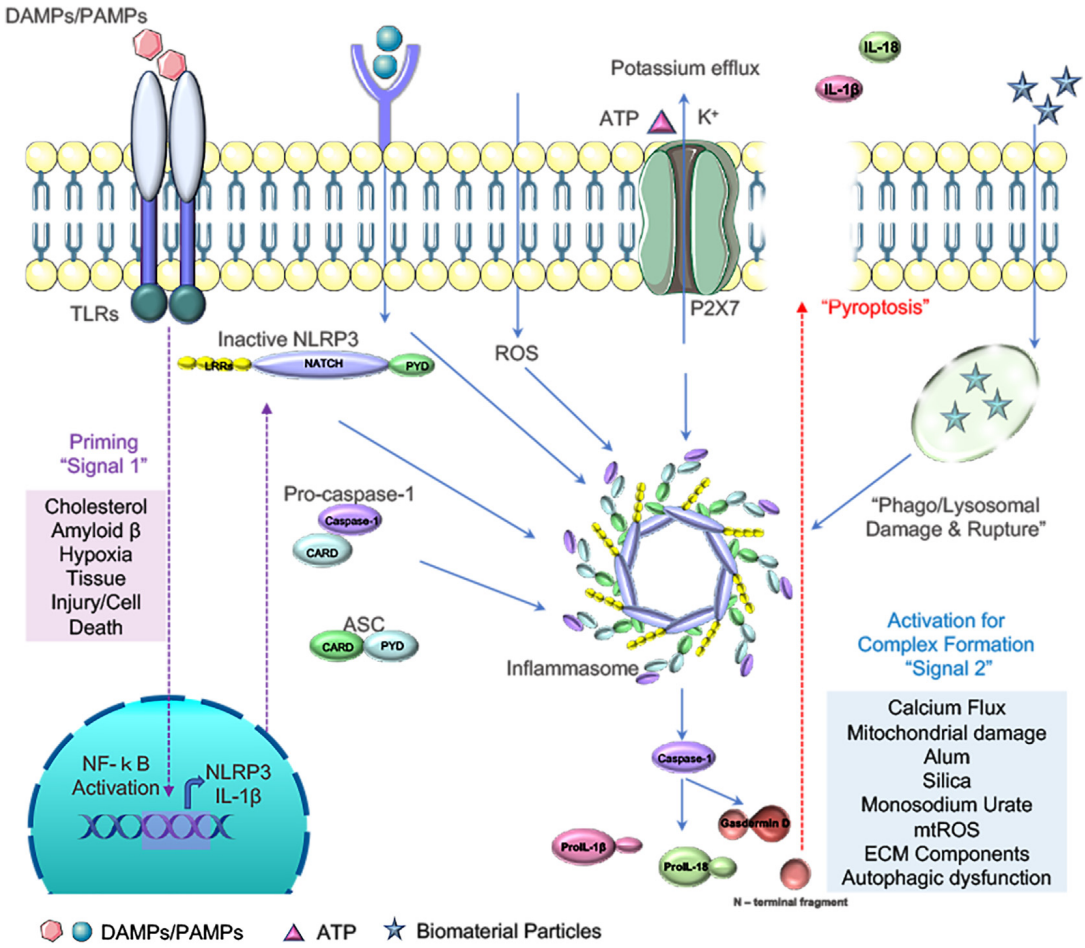



**Figure 4. Inflammasome activation.** The exposure to PAMPs or DAMPs, leads to TLRs stimulation causing NF-κB activation. NF-κB will then promote the transcription of NLRP3, proIL-1β, and proIL-18 that remain inactive in the cytoplasm. This signal (‘‘Signal 1”) is a priming event. For NLRP3 inflammasome activation a second signal is required (‘‘Signal 2”) leading to the oligomerization of inactive NLRP3, ASC, and procaspase-1. This protein complex will then convert procaspase-1 to caspase-1, which leads to the production and secretion of the mature IL-1β and IL-18. Different mediators have been described as the second step of inflammasome activation: Extracellular ATP can induce K+/potassium efflux through a purinergic P2X7-dependent pore; PAMPs and DAMPs trigger the generation of ROS; Phagocytosed environmental irritants or biomaterials (nanoparticles or biomaterial degradation products) may lead to lysosomal rupture and release of their contents. All the above described factors induce NLRP3 inflammasome assembly and activation.

### 5.3. The inflammasome in the inflammatory response to biomaterials

Different host-derived molecules that are revealing of tissue injury will activate the NLRP3 inflammasome; these molecules include extracellular ATP, high mobility group protein B1 (HMGB1), different types of crystals, as uric acid or cholesterol and hyaluronan [115, 125]. The implantation process of a biomaterial causes injury to the cells and will consequently release danger signals that could activate the NLRP3 inflammasome (Fig. 1).

The activation of inflammasomes by implanted biomaterials is still poorly understood and requires a more in-depth investigation (Table 1). There are already studies in the literature exploring the activation of inflammasomes by gold nanoshells [126], silver nanoparticles [127] and chitin/chitosan [128] based in the quantification of IL-1β production as a measure of inflammasome activation. Malik *et al*. [129] have performed a more detailed study and demonstrated the involvement of the inflammasome in interactions between cells and biomaterials and in the progress of the foreign body response using NLRP3, ASC, NLRC4 and caspase-1 deficient mice. They have observed that microspheres of poly(methyl metacrylate) (PMMA) can stimulate the NLRP3 inflammasome and cause the formation of an inflammatory exudate that depends on the inflammasome components NLRP3, ASC and caspase-1, and lead to the formation of active caspase-1 and secretion of IL-1β. Reisetter *et al*. [130] have demonstrated with *in vitro* studies using macrophages that exposure to carbon black nanoparticles lead to inflammasome activation assessed by the cleavage of caspase-1 to its active form and subsequent IL-1β release. Lunov *et al*. [131] have shown *in vitro* that amino-functionalized polystyrene nanoparticles (PS-NH2), but not carboxyl-(PS-COOH) or non-functionalized particles, trigger NLRP3 inflammasome assembly and downstream release of pro-inflammatory IL-1β by human macrophages. Gómes *et al*. [132] have investigated *in vitro* the activation of the NLRP3 inflammasome by silica nanoparticles (SiNPs) and reported that SiNPs lead to the production of pro-inflammatory cytokines with the participation of NLRP3 inflammasome components. Caicedo *et al*. [133] studied the role of shape and size of Cobalt-Chromium-Molybdenum (CoCrMo) alloy particles on human macrophage phagocytosis and inflammasome activation and found that larger and irregular particles induce higher macrophage IL-1β production due to inflammasome activation.

The NLRP3 inflammasome has been implicated in the biological response to wear debris resulting from joint replacements (Fig. 5). The normal usage of joint replacements inevitably results in the generation of wear debris and the biological response to these particles is complex and often drives the process towards periprosthetic tissue destruction and implant loosening. Wear debris act as danger signals in tissues around loose implants and are recognized as such or after phagocytosis by several PRRs. This will induce the activation of the NLRP3 inflammasome pathway leading to the activation of the pro-inflammatory cytokine precursors pro- IL-1β and pro-IL-18 by caspase-1, these pro-inflammatory mediators present in the joint fluid will lead to the recruitment, differentiation and maturation of osteoclasts precursors and thus, bone resorption will predominate over osteogenesis at the

bone- implant interface eventually leading to the loosening of the implant [133-136]. Burton *et al*. [137] investigated both *in vitro* and *in vivo* the contribution of the NLRP3 inflammasome in peri-implant osteolysis using perturbations of caspase-1 and inflammasome components. They recognize the NLRP3 inflammasome as an important mediator of wear-induced osteolysis and as a potential beneficial target for the treatment of periprosthetic osteolysis.

Continued investigation into how biomaterials activate the inflammasome is therefore of great interest [138]. Biomaterial recognition by inflammasomes [127, 128, 139] comprises key pathways, that can be targeted to improve biomaterial-tissue integration and subsequent tissue repair [129].

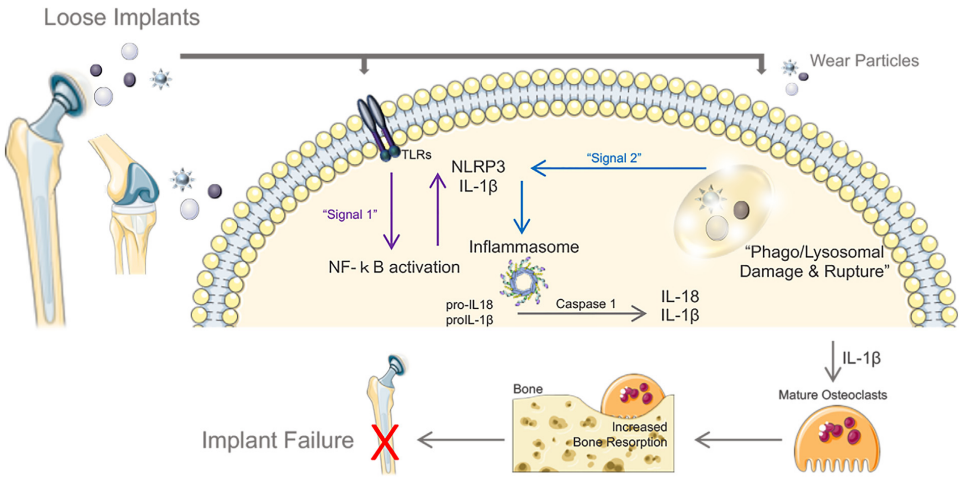



**Figure 5. Wear particles released from loose implants lead to inflammasome activation.** Wear particles are recognized as such or after phagocytosis (lysosomal rupture), by PRRs including TLRs and NLRs leading to the assembly of NLRP3 inflammasome. Once assembled the NLRP3 inflammasome cleaves pro-IL-1β into the active IL-1β. Secreted IL-1β can promote the maturation of osteoclasts into bone-resorbing cells increasing bone resorption and consequently impairing implant function.

**Table 1. Selected studies of the inflammasome role in the inflammatory response to biomaterials.**

| Biomaterial | Applicability | Pathways | Inflammasome | Cell/Immune Response |
|---|---|---|---|---|
| Gold nanoshell-enabled photothermal therapy (NEPTT) | Cancer therapies | Release of ATP, ADP and uric acid from tumor cells treated by NEPTT | NLRP3 | Induce the production of IL-1β [126] |
| Silica | Cosmetic; food and medical products | ROS production; Endosomal rupture; Leakage of cathepsin B | NLRP3 | Recruitment of inflammatory cells; IL-1β secretion [140-143] |
| Quantum Dots | Diagnostic and Therapeutic tools | Mitochondrial (mtROS) production | NLRP3 | Hepatocyte pyroptosis, liver inflammation [144] |
| Hydroxyapatite (HA) | Bioceramics in orthopaedics surgery | K+ efflux; ROS generation; lysosomal damage | NLRP3 | Stimulate the secretion of the pro-inflammatory cytokines-IL-1β and IL-18 [145]; |
| Microspheres of poly (methyl methacrylate) (PMMA) and Silicone discs | Wide variety of applications for biomedical research and life sciences | Clustering of lipid rafts resulting in nonspecific cytosolic aggregation of ITAM and activation of a Syk kinase pathway | ASC, Caspase 1 and AIM2 | Foreign Body response (Coll production; Leukocyte recruitment) 129];[146] |
| Mesoporous silica micro-rod (MSR) scaffold modified with PEG, PEG-RGD and PEG-RDG groups | Biomaterial scaffold-based vaccines | ROS production; Endosomal rupture | NLRP3 | MSR stimulated IL-1β production *in vitro* and played a key role in regulating immune cell infiltration *in vivo* [147] |
| Aluminium | Adjuvants in human and animal vaccines | Membrane disruption with resultant K+ efflux | NLRP3 | IL-1β and IL-18 production [148] |
| Calcium supplemented - Biphasic calcium phosphate (BCP/Ca) particles | Bone repair | Inhibition of ATP/P2X7R interactions | NLRP3 | Down-modulated, IL-1β and IL-18 synthesis [149] |
| Chitosan and Chitin | Regenerative medicine | Lysosomal rupture, K(+) efflux, ROS | NLRP3 | Higher doses of chitosan activated the inflammasome pathway, leading to an increase IL-1β and $PGE_2$ release [128, 139, 150] |

### 5.4. The inflammasome as a bridge between inflammation and regeneration

The immune system is of primary importance in orchestrating a correct repair process [58, 59], and since inflammasomes are involved in the innate immune response, it is expected that they have a key role in tissue repair/regeneration. The activation of the inflammasome and thus of caspase-1 has surprising consequences: it not only induces the inflammatory response through the activation and secretion of pro-inflammatory cytokines, but it also has an important role in the regulation of the extracellular levels of specific proteins, such as basic fibroblast growth factor (FGF-2), that are clearly involved in the processes of tissue repair and cytoprotection [151].

There are already some evidences described in the literature on the role of inflammasomes in tissue repair/regeneration. Recent studies using a murine skin wound repair model have interestingly establish a clear bridge between inflammation and tissue repair through the NLRP3 pathway. Weinheimer-Haus *et al*. [152] used a murine wound repair model in mice deficient in NLRP3 and caspase-1 and observed that these animals exhibited a reduced inflammatory response at day 5 following wounding with reduced levels of the pro-inflammatory cytokines IL-1$\beta$ and TNF-$\alpha$ together with reduced neutrophil and macrophage accumulation when compared to wild-type (WT) animals. It was also observed in the knockout (KO) mice a delay in wound healing when compared with WT mice. To assess whether loss of IL-1$\beta$ in wounds of NLRP3-KO mice was responsible for the defects observed in wound healing the authors performed a rescue experiment in NLRP3-KO mice treating the wounds with recombinant IL-1$\beta$ and concluded that treatment IL-1$\beta$ exhibited a trend of accelerated re-epithelialization. Taken together, these findings indicate that the NLRP3 inflammasome contributes to the early inflammatory phase following skin wounding and is important for efficient healing. Ito *et al*. [153] used WT and NALP3-KO and ASC-KO mice, and the overall conclusion of the study was that wound repair in mice was significantly impaired in NALP3 and ASC-KO mice when compared to the WT. The authors concluded that the genetic deficiency of NALP3 decreased the expression of pro-inflammatory cytokines together with a reduced inflammatory response at the skin wound site, resulting in impairment of wound repair, being similar results obtained using an inhibitor of NALP3. In addition, this study revealed that topical treatment with adenosine triphosphate (ATP), which is a ligand of NALP3, up-regulated the expression of pro-inflammatory cytokines at the wound site and accelerated wound healing in the WT mice. The authors demonstrated that the NALP3 pathway activation is involved in wound repair via upregulation of the inflammatory response, and the topical use of ATP promoted skin wound closure through the upregulation of the inflammatory response in the early wound-healing stage.

A very interesting study of liver regeneration using NLRP3-KO mice revealed that deficiency of NLRP3 signaling impairs liver regeneration. The activation of inflammasomes in the liver was induced after 70% partial hepatectomy. The liver-to-body weight ratio was significantly decreased in NALP3-KO mice when compared to WT mice after partial hepatectomy, and the expression of pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6) was decreased in the remnant liver of NALP3-KO mice compared to WT mice. In addition, treatment with ATP increased the liver-to-body weight ratio in WT mice. These results indicate that NALP3 signaling is required for the induction of an inflammatory response and the improvement of liver regeneration after partial hepatectomy [154].

Taken together, these results directly link inflammation to protective and regenerative processes being the inflammasome now considered as an attractive target to control tissue regeneration [155]. Thus, the understanding of the signaling that is elicited by inflammasome can be employed to improve healing [156].

There is extensive interest in the discovery of effective approaches that selectively inhibit the NLRP3 inflammasome pathway because this inflammasome is involved in a wide range of important processes from inflammation to tissue repair [157-161]. Therefore, modulation of nlrp3 activity is an important target to develop effective strategies for biomaterial integration which represents a rather important challenge in the biomedical research and clinical medicine [162, 163].

## 6. Chitosan

Chitosan (Ch), is a linear polysaccharide, obtained by deacetylation of chitin and is composed of (1→4)-2-acetamido-2-deoxy-β-D-glucan (D-Glucosamine) units, and it is the most widely used natural polymer after Coll. Chitin or poly (β-(1→4)-*N*-acetyl-D-glucosamine) is synthetized by living organisms, being the structural component of the shells of crustaceous, cell walls of fungi and exoskeletons of insects. However, due to poor solubility of chitin, practical applications are extremely reduced. The degree of deacetylation (DDA) of Ch, is related with the number of amino groups along the chain and it is calculated as the ratio of D-glucosamine to the sum of D-glucosamine and *N*-acetyl D-glucosamine. Chitosan should contain at least 60% of D-glucosamine residues (DDA of 60%), usually DDA fluctuates between 60% and 100% and its molecular weight (MW) from 300 to 1000 kDA [164]. Instead of DDA, some authors use the degree of acetylation (DA) to characterize Ch-based products, which represents the proportion of N-acetyl-D-glucosamine units with

respect to the total number of units. The DA allows to define the two terms chitin and Ch. So, in the case of Ch, DA is considered to be below 50% [165]. The DDA or DA is a structural parameter which influences physicochemical properties such as the MW [166] and also the biological properties, such as biodegradation by lysozyme [167], wound-healing properties [168] and immune response [169].

The amino groups in Ch chain differentiate Ch from chitin, offering to the polymer several peculiar properties. The amino groups of the D-glucosamine could be protonated making the Ch soluble in acidic solutions [170]. The amino groups along the Ch structure allow particular interactions with proteins, cells and living organisms. Due to its unique characteristics, as it is nontoxic, mucoadhesive, biocompatible and biodegradable, Ch has been used to several biomedical applications, such as drug delivery, gene therapy [171], vaccine adjuvants [172] and tissue engineering [173], with a variety of shapes and sizes. To date, 23.739 articles related to term Ch are indexed by Pubmed, and 8 Ch-based products that are currently under clinical trials with the status “recruiting/active, not recruiting” (Table 2), can be found at https://www.ncbi.nlm.nih.gov/pubmed/ and https://clinicaltrials.gov, respectively, demonstrating that it is a very active area which will yield many promising biomedical applications.

**Table 2. List of clinical studies containing the search term "chitosan" according to clinicaltrial.gov (accessed 15.01.2019).**

| Study Title | Conditions | Interventions | Location | Phase |
|---|---|---|---|---|
| Study of Chitosan for pharmacologic manipulation of AGE (Advanced Glycation Endproducts) levels in prostate cancer patients | Prostate cancer | Drug: Chitosan | United States of America (NCT03712371) | Phase 1 Phase 2 |
| Chitosan nerve tube for primary repair of traumatic sensory nerve lesions of the hand | Traumatic lesion of sensory nerves of the hand | Device Chitosan nerve tube in addition to gold standard therapy Procedure: Gold standard primary microsurgical repair | Germany (NCT02372669) | Not applicable |
| Chitosan nanoparticles, chlorhexidine gluconate, as intra canal medicaments in primary teeth | Necrotic pulp | Drug: Chitosan nanoparticles gel Drug: Chitosan gel Drug: Chlorhexidine gluconate | Egypt (*NCT03588351*) | Not applicable |
| The effect of peri-implant surgery and chairside supportive post-surgical peri-implant therapy | Periimplantitis Peri-implant mucositis | Device: Chitosan brush | Data not available (NCT03421717 | Not applicable |
| Treatment of Mild to Moderate Peri-implantitis Using an Oscillating Chitosan Device | Peri-Implantitis | Device: Labrida BioClean Other: Titanium curettes | Norway *(NCT03373448)* | Not applicable |
| Functional Chewing Gum in Reduction of Gingival Inflammation | Gengivitis | Device: Functional Chitosan Chewing Gum Device: Control Chewing Gum Behavioral: Oral hygiene measures | United States of America (NCT0323762) | Not applicable |
| Dental Extractions in Patients Under Dual Antiplatelet Therapy | Blood coagulation disorders | Device: HemCon Dental Dressing Device: Oxidized Cellulose Gauze | Brazil (NCT02918045) | Not applicable |
| Dental extractions in patients under dual antiplatelet therapy | Blood coagulation disorders | Device: HemCom dental dressing Device: Oxidized cellulose gauze | Brazil (*NCT02918045)* | Not applicable |

Table 2 (continuation)

| Study Title | Conditions | Interventions | Location | Phase |
|---|---|---|---|---|
| RAVE: Radial Artery Vascular Complication and Resource Utilization | Angiogram<br>Percutaneous<br>Coronary Intervention | Device: SoftSeal®-STF hemostatic pad<br>Device: VascBand™Hemostat | US<br>(NCT03522077) | Phase 4 |

### 6.1. Immunomodulatory properties of Ch

Modulating the immune system using immunomodulatory biomaterials may support the development of therapies that promote both systemic and local pro-regenerative immune responses, ultimately stimulating tissue repair. Chitosan exhibits different immunostimulatory behaviors, depending on their physical and chemical properties (such as molecular weight and DDA or DA). Chitosan products could lead immune cells to release pro– or anti-inflammatory mediators. The release of these different mediators can have a substantial impact on the performance of the different biomedical devices where Ch is used. Biodegradable Ch particles have been shown to promote the release of pro-inflammatory cytokines, IL-1β, TNF-α and chemokines associated with M1 macrophage phenotype [150, 174, 175]. Castro *et al*. showed that Ch/poly(γ-glutamic acid) nanoparticles with a DA of 11% (i.e., 89% glucosamine and 11% N-acetyl glucosamine) were able to modulate macrophage phenotype from a M2 macrophage polarization towards a M1 macrophage pro-inflammatory profile, decreasing the expression of CD163 and promoting the release of IL-12p40 and TNF-α [176]. We described that 3D Ch scaffolds with a DA of 15% induce a typical M1 macrophage response, leading to an increase of M1 surface markers (CCR7+) and pro-inflammatory cytokines (IL-6 and TNF-α), in an *in vivo* study [169]. On the other hand, Ch can also induce the release of anti-inflammatory cytokines, leading to an anti-inflammatory M2 macrophage phenotype [169, 174, 177, 178]. We showed in an *in vivo* model of inflammation that Ch 3D scaffolds with a lower DA (5%, i.e., 96% glucosamine and 4% N-acetyl glucosamine) induce a M2 macrophage phenotype, increasing the expression of the M2 marker CD206 and the release of anti-inflammatory cytokines, such as IL-4 [169]. Oliveira *et al*. demonstrated *in vitro* that 2D Ch films with a DA of 11% lead to an anti-inflammatory macrophage polarization, increasing the release of IL-10 and TGF-β1 and a pro-inflammatory dendritic cell stimulation, rising IL-1β and TNF-α [177].

Immune-modulating properties of Ch have been deeply studied for several years [179], however only recently intracellular pathways start to be clarified. The intracellular pathways associated to activation of immune cells by Ch-based products are the pathways that involve cGas-STING and NLRP3 inflammasome activation [139, 150, 172]. These results were observed following macrophage exposure to Ch with different DA and MW. NLRP3 activation and c-Gas-Sting pathways were recently linked in the context of macrophage infection by intracellular pathogens. Thus, macrophages use the same mechanisms to respond to Ch particle and intracellular pathogen uptake [180].

It is thus difficult to characterize Ch-based products as inducers of a pro- or anti-inflammatory response since both can have positive or negative effects depending on the type and severity degree of inflammation and also the conditions that needs to be resolved. Assessing the immunological effects of the chemical and physical properties of Ch in an *in vivo* context it is crucial to fully exploit the potential of this polymeric biomaterial in regenerative medicine.

### 6.2. Chitosan applications in tissue repair

During the development and fabrication of implantable materials for tissue engineering, several different aspects must be taken in account, namely biocompatibility, mechanical properties, scaffold morphology as well as healing and tissue replacement capacity. Chitosan has the ability to be shaped into different structures, such as microspheres [181], membranes [182], sponges [183], fibers [184], and porous scaffolds [185] for tissue engineering applications (Table 2). Due to its several remarkable properties, Ch appears as a pertinent candidate to substitute damage or missing tissues and to help in the repair of damaged organs [186].

For wound dressing, engineered materials should be non-toxic and non-allergenic, minimize scarring, provide scaffold for cell growth, allow gas exchanges, keep moist wound environment, protect the wound against secondary infections, adsorb wound exudates and accelerate tissue repair [186, 187]. Chitosan-based hydrogel is considered as an ideal material for wound healing due to its biodegradable, biocompatible, antimicrobial effects, and these properties could be modified by the addition of different natural or synthetic polymers. Chitosan-based hydrogel could also be used for the delivery of therapeutic drugs or growth factors to allow the achievement of a more effective treatment [188].

Nandi *et al* showed that Ch scaffolds alone or in combination with growth factors can be successfully used for bone defect healing. The similarities of the Ch scaffolds with natural bone tissue were useful to trigger the proliferation and differentiation of MSCs in osteoprogenitors cells [189].

**Table 3. Selected studies *in vitro*, *in vivo*, preclinical, clinical studies and commercially available of chitosan-based products for tissue engineering applications.**

| Chitosan product | Physical properties | Chemical properties | Applicability | Results |
|---|---|---|---|---|
| Chitosan | Chitosan Microspheres (CMS) with an ECM-mimicking nanofibrous structure | DA: 5% - 20%<br>MW: not provided | Cartilage tissue engineering | *In vitro* chondrocyte culture showed an enhanced cell attachment and proliferation and a macroscopic 3D geometrically shaped cartilage-like composite [190] |
| Chitosan/aloe | Membranes | DA:23.4%<br>MW:166kDa | Wound healing | A higher antimicrobial potential and good cell compatibility with primary human dermal fibroblasts when compared with chitosan membranes without aloe [191] |
| Chitosan | Porous 3D printed chitosan scaffolds | DA:25%<br>MW: 50-60kDa | Wound healing | In an *in vivo* test on rat models of diabetes, chitosan scaffolds promote the repair of skin, with an improved functionality, when compared with the commercial products [192] |
| 2-N,6-O-sulfated chitosan/recombinant human BMP-2 | Powder | MW: 5-8x$10^4$ | Bone healing | Induce a favorable immune microenvironment enhancing crosstalk between immune cells and stem cells undergoing osteogenic differentiation [193] |
| Porous Chitosan scaffolds | Scaffolds | MW: Low<br>DA:25%-15% | Bone healing | Porous chitosan alone and in combination with insulin like growth factor-1 (IGF-1) or BMP2 promote osteoblastic proliferation [189] |
| **Completed chitosan-related Clinical Studies** | | | | |
| Chitosan | Gel | Data not available | Chronic Wound | Significant reduction (>20%) of wound area in 90% of patients involved in the study [194] |
| Chitosan | Chitosan-based pads | Data not available | Haemostasis during percutaneous coronary procedures | Significant reduced haemostasis time in treatment group, when compared with control group [195] |
| **Commercial Products** | | | | |
| **Trademarks** | **Properties** | | | |
| Chitoflex® Hemcon | Control severe bleeding, while also offering an antibacterial barrier against a wide range of gram-positive and gram-negative organisms. | | | [188] |
| Tegasorb® 3M | Chitosan particles will swell while absorbing exudate and forming a soft gel. | | | |
| Chitopack C® Eisai | Cotton-like chitosan. Restoration of the normal subcutaneous tissue. | | | |
| Chitoseal®Abbott | Haemostatic function. | | | |

## 7. Bone

### 7.1. Bone tissue and mechanisms of bone remodeling

Bone is a highly specialized mineralized connective tissue with crucial functions in the body, such as support and protection of soft tissues, locomotion, mineral storage and harboring of bone marrow [196]. Structurally, bone is composed of two components: cortical and trabecular bone [197]. At the molecular level, bone tissue is constituted by an ECM composed by an organic phase and a mineral phase. The organic phase of bone matrix is mainly constituted by type I of Coll, and a panoply of non-collagenous proteins (e.g. proteoglycans, osteocalcin, osteopontin), while the mineral phase is constituted by hydroxyapatite, crystals of calcium and phosphate [198]. The bone cellular fraction is composed by bone-lining cells, osteoblasts, osteocytes, osteoclasts and resident populations of immune cells, designated as osteomacs (a resident macrophage population) [197, 199].

Osteoclasts are the only cells that are known to be able to resorb bone. These cells are derived from mononuclear precursor cells of the monocyte-lineage. Osteoclast formation is regulated by receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). RANKL is critical for osteoclast formation and M-CSF is required for their proliferation [197, 200]. Osteoclasts activation and resorption are regulated by the ratio of RANKL and osteoprotegerin (OPG), IL-1 and IL-6, parathyroid hormone, 1,25-dihydroxyvytamin D, and calcitonin [201, 202]. RANKL is produced by osteoblasts, osteoblasts precursors and T cells activated to stimulate osteoclast recruitment and activation.

The osteoprogenitor cells give rise to and maintain the osteoblasts that synthesize new bone matrix, the osteocytes within bone matrix that support bone structure, and the protective lining cells that cover the surface of quiescent bone. Bone marrow contains a small population of mesenchymal stromal cells (MSCs) that are capable to differentiate to lineages of mesenchymal tissues, like bone, cartilage, fat or fibrous connective tissues [203]. Osteoblast precursors change from spindle-shaped osteoprogenitors to large cuboidal differentiated osteoblasts on bone matrix surfaces. Active mature osteoblasts have large nuclei, enlarged Golgi, extensive endoplasmic reticulum, secrete type I Coll and other matrix proteins [197].

The bone lining cells (BLCs) are post-mitotic flat osteoblast lineage cells lining bone surface. There are no known markers to characterize BLCs or techniques to isolate them. BLCs are

pointed as a source of active osteoblasts [204-206] and one of their function is related with the removal of demineralized matrix prior to bone formation [207].
Osteocytes are the terminally differentiated osteoblasts that remained entrapped in bone matrix. Osteocytes major functions are to support bone structure, metabolism and are mechanosensitive. Osteocytes lie within lacunae within mineralized bone and present extensive filopodial processes [208]. Osteocytes could live decades in human bone. Empty lacunae in aging bone means that osteocytes may have suffered apoptosis [209].

Osteal macrophages (Osteomacs) are tissue resident macrophages in the endosteal and periosteal space that have a crucial role in bone homeostasis, supporting bone formation and mediating parathyroid hormone-dependent bone repair [210]. Osteomacs have been shown to support osteoblast differentiation and mineralization *in vitro*, and to be involved in intramembranous bone healing after fracture [211] and physiologic skeletal remodeling [212]. In addition, these cells also help the maintenance of hematopoietic stem cells niches [213].

During development, bone is formed by either intramembranous or endochondral ossification. Flat bones are formed by intramembranous ossification and long bones are formed by endochondral ossification [214]. Bone is an active tissue that is continuously being remodeled to remove old and microdamaged bone and replaced with new bone, through the concerted actions of different cells, which include bone-resorbing cells, the osteoclasts and bone-forming cells, the osteoblasts, being the osteocytes the mechanosensors and orchestrators of the bone remodeling process [215, 216]. The remodeling of this complex tissue can be divided in four sequential phases: (i) Activation (activation and recruitment of mononuclear monocyte-macrophage osteoclast precursors from the circulation), (ii) Resorption (bone resorption is mediated by osteoclasts secreting tartrate-resistant acid phosphate, cathepsin K, MMP9 and gelatinase from cytoplasmic lysosomes to digest the organic matrix), (iii) Reversal (transition phase from bone resorption to bone formation) and (iv) Formation/termination (matrix synthesis by osteoblasts) (Fig.6) [197]. The bone remodeling process takes several weeks to occur and is performed by clusters of bone-resorbing osteoclasts and bone-forming osteoblasts arranged in a structure known as “basic multicellular units” (BMUs) [217]. The main recognized functions of bone remodeling include preservation of bone mechanical strength, bone health and mineral homeostasis.

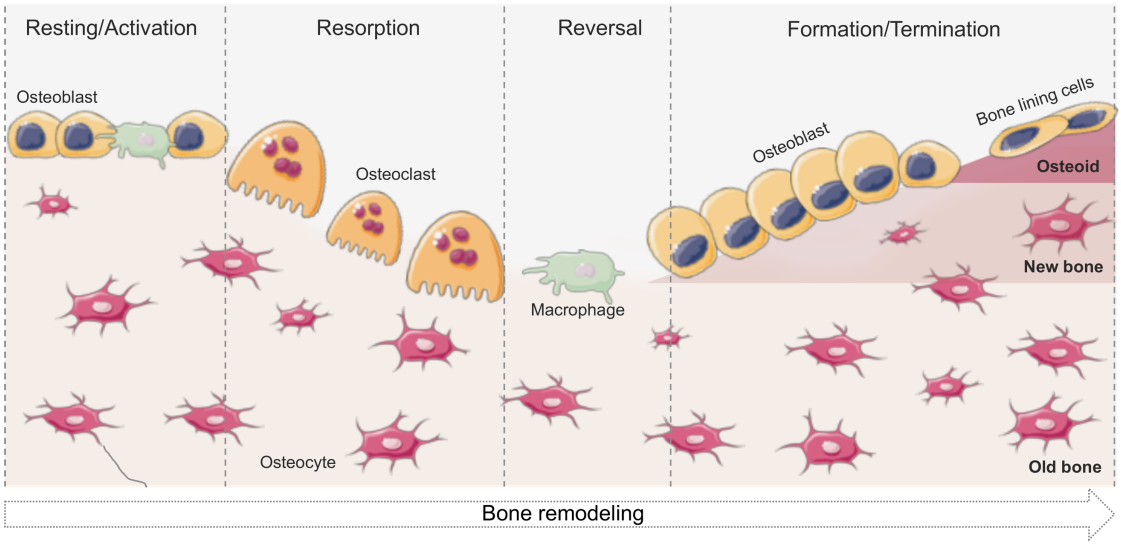



**Figure 6. Bone remodeling.** The remodeling cycle is composed for 4 sequential phases, namely, activation/resting, resorption, reversal, formation/termination. Resting/activation phase is characterized by activation and recruitment of mononuclear monocyte-macrophage osteoclast precursors from the circulation. Resorption phase is mediated by osteoclasts starting the digestion of the organic matrix. In the reversal phase occurs the transition from bone resorption to bone formation. During formation/termination phase, osteoblasts are recruited to the resorbed area starting matrix synthesis. Osteoblasts that get trapped in the bone matrix during bone formation become osteocytes.

### 7.2. The role of the immune system in bone fracture repair

The repair of bone fractures recapitulates bone development and can be considered a form of tissue regeneration. Fracture healing involves numerous signaling pathways and cell types [218, 219]. It follows one of two processes: (i) primary or direct fracture repair and (ii) secondary fracture repair. Primary fracture requires proximity and rigidly stable conditions leading to little or no inflammation [220]. Secondary fracture repair is the most common form and consists of both endochondral and intramembranous bone [221] with highly regulated interactions between immune cells, MSCs, osteoblasts and osteoclasts [222, 223]. Intramembranous ossification is characterized by direct differentiation of MSCs into the osteoblastic lineage, that will deposit bone matrix and promote its mineralization into mature woven bone. On the other hand, endochondral ossification requires the synthesis of a cartilage template by MSCs, the bone callus, being mineralized into woven bone and later replaced by mature bone [224, 225]. Due to the key role of the immune system in fracture repair Loi *et al*. suggested that the so-called “diamond concept” of fracture healing [226] should be modified to included immune cells and their mediators [220]. It involves three overlapping phases: (i) inflammatory; (ii) repair and (iii) remodeling phases (Fig. 7). During these processes innate and adaptive immune cells, cytokines and chemokines have

a significant role to play, since without an immune response, bone will not heal properly [222].

**(i) Inflammatory phase**

After bone injury, a local disruption of blood supply and soft tissue occurs. Vascular disruption results in clot formation to provide homeostasis and the establishment of a hematoma that will act as the future template for callus formation. Blood and immune cells, including platelets, neutrophils and monocyte/macrophages are recruited to the injury site [225]. T and B-lymphocytes are recruited 3 days after injury and are reduced with cartilaginous callus formation [227]. The acute inflammatory response peaks within the first 24h and is normally complete after 14 days [219]. At this stage, mediators responsible for inflammation resolution, such as SPMs are reported to be secreted by neutrophils and macrophages, promoting the end of the inflammatory response [228]. The initial pro-inflammatory response involves the secretion of TNF-$\alpha$, IL-1, IL-18, IL-6, and IL-11 [225]. TNF-$\alpha$, IL-1, and IL-6 are essential for the inflammatory response that triggers osteogenesis. In fact, TNF-$\alpha$ and IL-1 exhibit a biphasic response, with high levels expressed immediately following injury, decreasing within 72 hours. Both cytokines at 3 - 4 weeks after injury exhibit again high levels that may correspond to the remodeling phase [229]. These factors will lead to the recruitment of inflammatory cells and MSCs and promote angiogenesis. Simultaneously, recruited osteoprogenitor cells produce BMPs which, in coordination with other factors, promote the local recruitment and osteogenic differentiation of MSCs [230]. The hematoma is reorganized and there is a deposition of a fibrin thrombus. As capillaries invade the thrombus, granulation tissue replaces the fibrin clot. Neutrophils and macrophages remove dead cells and debris [231] and promote the recruitment of MSCs (periosteum, bone marrow and systemic circulation) [225]. These cells have an immunosuppressive function which helps to resolve the inflammation and prepare the local for the next phase of healing.

**(ii) Repair phase: cartilaginous and bony callus formation**

**-Cartilaginous callus formation**

During the inflammatory phase, MSCs are recruited to the site of injury and undergo chondrogenic differentiation. The decrease of mechanical stability at the fracture site promotes chondrogenesis. The granulation tissue is replaced by a semi-rigid fibrocartilaginous callus that provides mechanical support. In the initial phase, cartilaginous callus is avascular; but, as healing proceeds, the callus is invaded by endothelial cells

promoting angiogenesis. This induces terminal differentiation of chondrocytes, resulting in hypertrophy and the production of mineralized cartilaginous matrix [232-234].

**-Bony callus formation**

Upon calcification of the fracture callus, osteoprogenitor cells are recruited from the periosteum, bone marrow, vasculature, and surrounding tissue to initiate osteogenesis and the deposition of bone onto the calcified cartilage. During this phase osteoclasts are activated to resorb the cartilaginous callus, resulting in the transformation of soft callus into a hard callus, with a mineralized matrix produce by osteoblasts. Woven bone formation is initiated, providing greater stability than the fibrocartilaginous callus. Immune cells, like macrophages T and B cells play a decisive role during mineralization [224, 232].

**(iii) Remodeling phase**

The remodeling phase correspond to the last stage of fracture repair. The woven bone (Coll fibrils are randomly oriented) within the callus is replaced with laminar bone (Coll fibrils are clustered in parallel arrays), consisting of highly organized matrix of Coll fibers, therefore restoring the original structure and function of the bone. This process is driven by osteoclast-mediated bone resorption followed by osteoblast-mediated bone formation [219, 223, 235].

### 7.2.1. Immune dysfunction in bone repair

A healthy immune system is fundamental for a precise fracture healing. Co-morbidities with chronic inflammation such as rheumatoid arthritis (RA) [236], diabetes mellitus [237], obesity and systemic lupus erythematosus (SLE) [238] are associated with a poor fracture healing [239, 240]. Pro-inflammatory cytokines such as IL-1 and TNF-$\alpha$ that are highly expressed in these diseases strongly induce osteoclastogenesis, impairing fracture healing [241]. In a diabetic model of fracture healing, TNF-$\alpha$ increased chondrocyte apoptosis and premature loss of cartilage diminishing bone formation [242]. Hence, a well orchestrate balance between anti and pro-inflammatory mediators is advantageous during bone fracture healing.

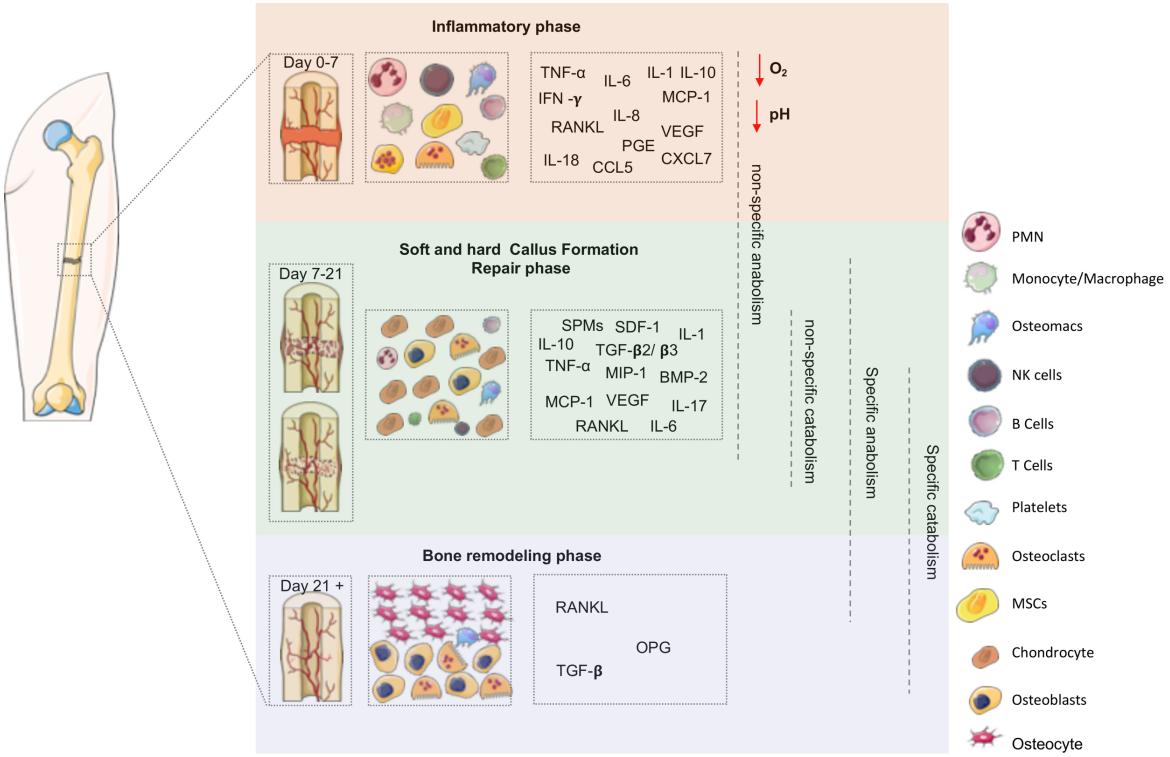



**Figure 7.** Role of immune cells during fracture repair. Bone fracture healing can be viewed as a three-stage process: (i) inflammatory, (ii) repair and (iii) remodeling phases. These three phases are characterized by different cell types, cytokines, chemokines and growth factors that are involved. (i) The inflammatory phase starts with the activation of blood coagulation cascade; formation of provisional fibrin matrix (hematoma); release of danger signal molecules and activation of local macrophages followed by an acute inflammation that is characterized by the recruitment of neutrophils and macrophages; clearance of necrotic tissue and provisional matrix; production of pro-inflammatory cytokines, chemokines, growth factors and the recruitment and activation of MSCs, osteoprogenitors, fibroblasts. Inflammatory phase occurs at low pH and oxygen. (ii) Repair phase (soft and hard callus formation) proliferation of progenitor cells; deposition of immature fibrotic extracellular matrix; angiogenesis; differentiation of progenitor cells into chondrocytes and production of fibrocartilage; differentiation of progenitor cells into osteoblasts and production of woven bone; fracture stabilization; apoptosis of chondrocytes; woven bone deposition on cartilage scaffold. (iii) Remodeling phase, bone repair ends with resorption of cartilage and woven bone and restoration of harversian system. Immune cells play important roles throughout this process; however, a majority of their activity occurs during early stages of fracture healing. IL-interleukin; IFN-interferon; MCP - monocyte chemoattractant protein; RANKL - Receptor activator of nuclear factor kappa-B ligand; PGE-prostaglandin; VEGF-vascular endothelial growth factor; CXCL-chemokine (C-X-C motif) ligand; SPMs-specialized proresolving mediators; TGF-transforming growth factor; MIP-macrophage inflammatory protein; BMP-bone morphogenetic protein; OPG-osteoprotegerin.

### 7.2.2. Macrophages and bone repair

Interplay between macrophages and bone cells is critical to bone formation and repair. Resident macrophages, the osteomacs, are present all over osteal tissue [210]. Osteomacs

differ from osteoclasts in expression of Siglec 1 (CD169) and its depletion compromised bone repair in two different models of bone injury, intramembranous (tibial injury) or endochondral (internally-plated femoral fracture model) [243]. Upon bone injury and similar to other tissues, resident macrophages release several factors, such as, cytokines, chemokines and growth factors to recruit other immune cells to engage the tissue repair. Through the release of MCP-1, TNF-$\alpha$, IL-1, IL-6, CCL2, and RANTES (Regulated on Activation Normal T Cell Expressed and Secreted) macrophages stimulate the recruitment of MSCs in the early phase of fracture healing [229, 244]. It has been demonstrated the anabolic effect of macrophages on osteogenesis [245-247]. Monocytes and macrophages support osteoblast differentiation and proliferation. Schlundt *et al*. showed that for a fracture healing to successfully occur, a fine balance in M1/M2 macrophage function is required [41].

### 7.3. Therapeutic approaches through a biomaterial inflammatory - centered methodology

Fracture healing is a highly complex process that involves different cells and released factors, tissue matrix and the immune system. As described earlier, immune cells play a critical role in bone fracture healing being the first responders and the initiators of the repair processes. The gold standard therapeutic approaches include the use of autografts, allografts and xenografts. Some problems are associated to these strategies, such as the requirement of a second donor, in the case of autografts prolonged operative times and potential immune response in the case of allograft and xenografts. Thus, current therapies for the treatment of non-healing bone fractures and bone disease are inadequate [239]. Consequently, a more workable, long term treatment strategy is required. To that end, bone graft substitutes are being engineered to help in the surgical revision of non-healing fractures [248]. Depending on the severity of the fracture, different approaches for bone repair could be used: (i) synthetic scaffolds alone, (ii) scaffolds combined with active molecules and (iii) cell-based combination products with cells from different sources [248]. Indeed, scaffolds provide the structural support for tissue repair.

The ideal scaffold for bone tissue engineering should be able to promote (i) osteoinduction, (ii) osteoconduction, (iii) osteogenesis and (iv) mechanical stability [249]. The functionalization of scaffolds should involve controlled release of active molecules and combine scaffold degradation with new bone formation [220, 250-252]. Biomaterial-immune system interactions are being increasingly recognized as a crucial factor influencing tissue repair. The development of novel materials that can diminish the FBR and improve

engraftment is a new trend in the tissue engineered products [76]. It is described that the host immune reaction following biomaterial implantation of a bone substitute material determines the success of bone repair [253]. More recently, increasing evidence has demonstrated that injuries with poor healing outcomes are associated with delayed M1-to-M2 transition [254, 255].

A novel therapeutic approach in the field is to replicate the sequential release of polarizing macrophage factors that promote the classical activation into M1-macrophage followed by the switch to M2 phenotype. Spiller *et al*. designed scaffolds for bone regeneration based on decellularized bone for a short release of IFN-γ to promote M1 phenotype, followed by a prolonged release of IL-4 to promote M2 phenotype. Results showed that these scaffolds were able to promote *in vitro* a sequential M1 and M2 polarization in primary human macrophages and *in vivo* exhibited increased vascularization in scaffolds releasing IFN, when comparing with controls [92]. It is described that M1 phenotype initiate angiogenesis while M2 macrophages promote vessel maturation [256]. Alhamdi *et al*. tested a biomimetic calcium phosphate coating (bCap) as a platform for local delivery to guide macrophage phenotype using old mice [257]. They observed that aging delayed bone healing due to alterations in bone cells and in the immune system. The bCap was used to separate physically and temporally the pro-inflammatory stimulus IFN-γ from the pro-reparative mediator simvastatin (described to have a modulatory effect on the inflammatory mediators [258]). The results obtained showed a sequential M1-to-M2 transition in THP-1 cells and in macrophages from old mice [257]. Li *et al*. showed in a 3D printed scaffold designed for the repair of massive bone defects that the release of IFN-γ and silicon ions promoted sequential M1 to M2 polarization improving angiogenesis [259]. Zheng *et al*. using a decellularized bone matrix (DBM) to delivery IL-4 demonstrated that a proper delivery of IL-4 can generate an accurate immunomodulation via the synchronized involvement of M1 and M2 macrophages resulting in a pro-healing microenvironment with a significantly osteogenesis and angiogenesis [260].

The low success rates achieved in rebuilding human bone tissues may be due in part to the propensity for therapeutic strategies to target later processes in fracture healing and repair, such as MSCs differentiation [261]. Since the immune system is a highly plastic network that acts as a guardian of tissue integrity and is able to adapt to the nature of the local microenvironment [262], the modulation of the inflammatory signaling pathways using engineered biomaterials emerges as a promising new strategy to promote bone repair.

### 7.4. The socio – economic impact of bone fractures

Bone fractures are one of the most common organ injuries in humans. Bone fracture healing is a complex process that under healthy circumstances occurs without scar tissue formation. However, due to several causes such as fractures above critical size and co-morbidities, i.e. systemic inflammations, tumors, genetic factors and poor lifestyle (i.e. smoking or alcohol abuse), 5 - 10% of bone fractures do not heal properly leading to non-union fractures [239, 248]. Fracture treatments and bone repair solutions generate important revenues. The global orthopedic market was valued at approximately $52.8 billion USD in 2017, driven by the growth of the aging population. The orthopedic market includes, surgical power tools, all products used to repair fractures internally or externally, spinal fusion and orthobiologics that include different strategies to heal bone defects more quickly, such as active molecules, stem cells or demineralized bone grafts. The market is expected to present an annual growth rate of 3.8% until 2023 [248, 263]. The current “gold standard” treatment for slow or incomplete bone healing is to perform bone grafting. Nonetheless, there are limitations to bone grafting regarding the limited volume of bone that can be harvested and also donor site morbidity [264, 265]. Therefore, a more workable long-term treatment strategy is required. To that end, biomaterials are being engineered to help impaired fracture healing [248].

## Thesis Outline

This thesis is divided in seven chapters that provide a detailed review of literature, presents the developed work, and discuss the findings and their implications for the field.

**Chapter I** provides a general overview regarding the progress in biomaterials field and their applications through the years. The inflammatory response to biomaterials is described along with the new trend in biomaterials development (immunomodulatory materials); the relevance of a well-orchestrated inflammatory response for tissue repair and the key role of macrophages during tissue healing. A general information about Ch-based products and their biomedical applications is also provided with emphasis on the immunomodulatory properties of Ch. Finally, the role of immune system on bone repair is illustrated together with the major limitations on bone fractures treatments, the socio-economic impact of bone fractures worldwide and new approaches for the improvement of bone healing are also highlighted.

**Chapter II** offers a comprehensive explanation of the main aims and motivations of the work developed in this thesis.

**Chapter III** is the first chapter of original data and describes the role of NLRP3 inflammasome in macrophage response to 3D Ch scaffolds with different DA (4% and 15%). The NLRP3 inflammasome activation pathway was recently linked to macrophage response to Ch-based products. Due to the lack of studies related with the role of NLRP3 inflammasome and macroscopic Ch-based products, in this chapter we explored the effect of 3D Ch scaffolds on NLRP3 inflammasome activation in primary mouse and human macrophages. Chitosan scaffolds were able to modulate NLRP3 inflammasome activation in mouse and human macrophages, an effect not dependent on the DA. This study allows us to start to dissect the role of NLRP3 inflammasome in macrophage response to macroscopic biomaterials.

**Chapter IV** presents the development of a novel immunomodulatory biomaterial. Resolvin D1 (RvD1), a pro-resolving mediator with relevant functions in the resolution of inflammatory responses, was incorporated in 3D Ch scaffolds with higher DA (15%) and lyophilized immediately. The developed material (Ch + RvD1 lyophilized); Ch scaffolds with fresh RvD1 solution (Ch + RvD1) were implanted *in vivo,* in a model of inflammation, the mouse air-pouch model. Each animal received an implant and Ch scaffolds alone were used as controls. Four days after materials implantation, the inflammatory exudates and Ch

scaffolds were recovered for analysis of macrophage cell markers; cytokines produced, and cell infiltrate in Ch scaffolds. The developed material was able to shift the macrophage phenotype transition from M1 to M2 polarization. The sequential transition of macrophage phenotype induced by the developed material offers a new strategy to improve tissue repair, since a M2 anti-inflammatory pro-reparative phenotype is associated to an amelioration of tissue healing outcome.

**Chapter V** describes the application of the immunomodulatory material previously developed in an *in vivo* model of tissue repair. As the results presented in chapter IV were very promising, herein we present the proof of concept of the developed material. The main aim of this work was verified if the Ch + RvD1 lyophilized could improve tissue healing. For that, a rat femoral defect model was used. Each animal was implanted with a Ch scaffold in the defect created in the right condyle of femurs and two months after injury, femurs were collected and analyzed for new bone and Coll formation. Non-operated animals were used as controls. The results showed that RvD1 has a positive effect on the new bone formation, since the new bone formed presented features that are linked with high quality and strength.

**Chapter VI** focuses on the development of a new strategy to improve the release time and the half-life of RvD1. A sustained release of RvD1 would improve the outcomes concerning tissue repair. Solid lipid nanoparticles (SLNs) were used as delivery system for RvD1. RvD1-loaded SLNs were developed and tested to assess its capacity to modulate the macrophage response to lipopolysaccharide (LPS). The developed nanoparticles allowed the decrease of pro-inflammatory cytokines in human macrophages primed with LPS. The results of RvD1-loaded SLNs were similar to the results obtained with fresh RvD1 free solution even when prepared a day before use, which suggest that the stability of this mediator was improved.

**Chapter VII** presents a general discussion of the results described in the previous chapters and suggestions of future work in this research area. We have decided to present a general discussion instead of detailed discussion because the latter is presented in each of the preceding chapters.

## REFERENCES

[1] L. Hench, Biomaterials, Science 208(4446) (1980) 826-831.

[2] W. Bonfield, M.D. Grynpas, A.E. Tully, J. Bowman, J. Abram, Hydroxyapatite reinforced polyethylene--a mechanically compatible implant material for bone replacement, Biomaterials 2(3) (1981) 185-6.

[3] J.W. LL Hench, Surface-active biomaterials, Science 226(4675) (1984) 630-636.

[4] J.M.P. L.L. Hench, I.D. Xynos, L.D.K. Buttery, Bioactive materials to control cell cycle, Materials Research Innovations 3(6) (2000) 313-323.

[5] L.R. Schoen FJ, Piehler HR., Pathological considerations in replacement cardiac valves, Cardiovasc Pathol. 1(1) (1992) 29-52.

[6] L.L. hench, Wilson, J.W., Clinical performance of skeletal prostheses, Chapman and Hall, London, UK, 1996.

[7] L.L. Hench, J.M. Polak, Third-generation biomedical materials, Science 295(5557) (2002) 1014-7.

[8] L.L. Hench, I. Thompson, Twenty-first century challenges for biomaterials, Journal of the Royal Society, Interface / the Royal Society 7 Suppl 4 (2010) S379-91.

[9] E.S. Place, N.D. Evans, M.M. Stevens, Complexity in biomaterials for tissue engineering, Nat Mater 8(6) (2009) 457-70.

[10] MarketsandMarkets.com, Biomaterials Market by Type of Materials & Application - Global Forecast to 2021. 2016).

[11] D.F. Williams, Definitions in biomaterials: proceedings of a consensus conference of the European Society for Biomaterials, Elsevier Science Limited, Chester, England, March 3–5, 1986, 1987.

[12] Definitions of Biomaterials for the Twenty-first Century, Proceedings of a Consensus Conference Held in Chengdu, People's Republic of China, June 11-12, 2018 (2018).

[13] J.M. Anderson, Inflammatory response to implants, ASAIO Trans 34(2) (1988) 101-7.

[14] J.M. Anderson, Biological Responses to Materials, Annual Review of Materials Research 31(1) (2001) 81-110.

[15] J.M. Anderson, A. Rodriguez, D.T. Chang, Foreign body reaction to biomaterials, Seminars in immunology 20(2) (2008) 86-100.

[16] R. Medzhitov, Origin and physiological roles of inflammation, Nature 454(7203) (2008) 428-35.

[17] S. Franz, S. Rammelt, D. Scharnweber, J.C. Simon, Immune responses to implants - a review of the implications for the design of immunomodulatory biomaterials, Biomaterials 32(28) (2011) 6692-709.

[18] G. Weissmann, J.E. Smolen, H.M. Korchak, Release of inflammatory mediators from stimulated neutrophils, N Engl J Med 303(1) (1980) 27-34.
[19] J.D. Andrade, V. Hlady, Protein adsorption and materials biocompatibility: A tutorial review and suggested hypotheses., Advances in Polymer Science 79 (1985) 1-63.
[20] J. Benesch, P. Tengvall, Blood protein adsorption onto chitosan, Biomaterials 23(12) (2002) 2561-8.
[21] M.B. Gorbet, M.V. Sefton, Biomaterial-associated thrombosis: roles of coagulation factors, complement, platelets and leukocytes, Biomaterials 25(26) (2004) 5681-703.
[22] M.R. Elliott, F.B. Chekeni, P.C. Trampont, E.R. Lazarowski, A. Kadl, S.F. Walk, D. Park, R.I. Woodson, M. Ostankovich, P. Sharma, J.J. Lysiak, T.K. Harden, N. Leitinger, K.S. Ravichandran, Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance, Nature 461(7261) (2009) 282-6.
[23] S. de Oliveira, A. Lopez-Munoz, S. Candel, P. Pelegrin, A. Calado, V. Mulero, ATP modulates acute inflammation in vivo through dual oxidase 1-derived H2O2 production and NF-kappaB activation, Journal of immunology 192(12) (2014) 5710-9.
[24] P. Pelegrin, Many ways to dilate the P2X7 receptor pore, Br J Pharmacol 163(5) (2011) 908-11.
[25] M.E. Bianchi, DAMPs, PAMPs and alarmins: all we need to know about danger, J Leukoc Biol 81(1) (2007) 1-5.
[26] S.N. Christo, K.R. Diener, A. Bachhuka, K. Vasilev, J.D. Hayball, Innate Immunity and Biomaterials at the Nexus: Friends or Foes, Biomed Res Int 2015 (2015) 342304.
[27] J. Lee, J.G. Jackman, J. Kwun, M. Manook, A. Moreno, E.A. Elster, A.D. Kirk, K.W. Leong, B.A. Sullenger, Nucleic acid scavenging microfiber mesh inhibits trauma-induced inflammation and thrombosis, Biomaterials 120 (2017) 94-102.
[28] K.A. Hooper, T.L. Nickolas, E.J. Yurkow, J. Kohn, D.L. Laskin, Characterization of the inflammatory response to biomaterials using a rodent air pouch model, Journal of biomedical materials research 50(3) (2000) 365-74.
[29] L. Tang, J.W. Eaton, Inflammatory responses to biomaterials, Am J Clin Pathol 103(4) (1995) 466-71.
[30] J.S. Duffield, The inflammatory macrophage: a story of Jekyll and Hyde, Clin Sci (Lond) 104(1) (2003) 27-38.
[31] D.M. Mosser, J.P. Edwards, Exploring the full spectrum of macrophage activation, Nature reviews. Immunology 8(12) (2008) 958-69.
[32] A. Mantovani, C. Garlanda, M. Locati, Macrophage diversity and polarization in atherosclerosis: a question of balance, Arterioscler Thromb Vasc Biol 29(10) (2009) 1419-23.

[33] S.F. Badylak, T.W. Gilbert, Immune response to biologic scaffold materials, Seminars in immunology 20(2) (2008) 109-16.

[34] A. Mantovani, A. Vecchi, P. Allavena, Pharmacological modulation of monocytes and macrophages, Curr Opin Pharmacol 17 (2014) 38-44.

[35] R.J. Miron, D.D. Bosshardt, Multinucleated Giant Cells: Good Guys or Bad Guys?, Tissue Eng Part B Rev 24(1) (2018) 53-65.

[36] Z. Xia, J.T. Triffitt, A review on macrophage responses to biomaterials, Biomed Mater 1(1) (2006) R1-9.

[37] R. Klopfleisch, F. Jung, The pathology of the foreign body reaction against biomaterials, Journal of biomedical materials research. Part A 105(3) (2017) 927-940.

[38] R. Klopfleisch, Macrophage reaction against biomaterials in the mouse model - Phenotypes, functions and markers, Acta biomaterialia 43 (2016) 3-13.

[39] T.A. Wynn, K.M. Vannella, Macrophages in Tissue Repair, Regeneration, and Fibrosis, Immunity 44(3) (2016) 450-462.

[40] Y. Okabe, R. Medzhitov, Tissue biology perspective on macrophages, Nature immunology 17(1) (2016) 9-17.

[41] C. Schlundt, T. El Khassawna, A. Serra, A. Dienelt, S. Wendler, H. Schell, N. van Rooijen, A. Radbruch, R. Lucius, S. Hartmann, G.N. Duda, K. Schmidt-Bleek, Macrophages in bone fracture healing: Their essential role in endochondral ossification, Bone 106 (2018) 78-89.

[42] C. Nathan, Metchnikoff's Legacy in 2008, Nature immunology 9(7) (2008) 695-8.

[43] A. Sica, A. Mantovani, Macrophage plasticity and polarization: in vivo veritas, The Journal of clinical investigation 122(3) (2012) 787-95.

[44] F.O. Martinez, S. Gordon, The M1 and M2 paradigm of macrophage activation: time for reassessment, F1000Prime Rep 6 (2014) 13.

[45] F. Ginhoux, J.L. Schultze, P.J. Murray, J. Ochando, S.K. Biswas, New insights into the multidimensional concept of macrophage ontogeny, activation and function, Nature immunology 17(1) (2016) 34-40.

[46] P.J. Murray, J.E. Allen, S.K. Biswas, E.A. Fisher, D.W. Gilroy, S. Goerdt, S. Gordon, J.A. Hamilton, L.B. Ivashkiv, T. Lawrence, M. Locati, A. Mantovani, F.O. Martinez, J.L. Mege, D.M. Mosser, G. Natoli, J.P. Saeij, J.L. Schultze, K.A. Shirey, A. Sica, J. Suttles, I. Udalova, J.A. van Ginderachter, S.N. Vogel, T.A. Wynn, Macrophage activation and polarization: nomenclature and experimental guidelines, Immunity 41(1) (2014) 14-20.

[47] L.C. Davies, S.J. Jenkins, J.E. Allen, P.R. Taylor, Tissue-resident macrophages, Nature immunology 14(10) (2013) 986-95.

[48] Q. Zhang, M. Raoof, Y. Chen, Y. Sumi, T. Sursal, W. Junger, K. Brohi, K. Itagaki, C.J. Hauser, Circulating mitochondrial DAMPs cause inflammatory responses to injury, Nature 464(7285) (2010) 104-7.

[49] T.A. Wynn, Cellular and molecular mechanisms of fibrosis, The Journal of pathology 214(2) (2008) 199-210.

[50] P. Laurent, V. Jolivel, P. Manicki, L. Chiu, C. Contin-Bordes, M.E. Truchetet, T. Pradeu, Immune-Mediated Repair: A Matter of Plasticity, Frontiers in immunology 8 (2017) 454.

[51] M.Z. Zhang, B. Yao, S. Yang, L. Jiang, S. Wang, X. Fan, H. Yin, K. Wong, T. Miyazawa, J. Chen, I. Chang, A. Singh, R.C. Harris, CSF-1 signaling mediates recovery from acute kidney injury, The Journal of clinical investigation 122(12) (2012) 4519-32.

[52] T. Lucas, A. Waisman, R. Ranjan, J. Roes, T. Krieg, W. Muller, A. Roers, S.A. Eming, Differential roles of macrophages in diverse phases of skin repair, Journal of immunology 184(7) (2010) 3964-77.

[53] R. Mirza, L.A. DiPietro, T.J. Koh, Selective and specific macrophage ablation is detrimental to wound healing in mice, Am J Pathol 175(6) (2009) 2454-62.

[54] T.A. Wynn, L. Barron, Macrophages: master regulators of inflammation and fibrosis, Semin Liver Dis 30(3) (2010) 245-57.

[55] T.J. Koh, L.A. DiPietro, Inflammation and wound healing: the role of the macrophage, Expert Rev Mol Med 13 (2011) e23.

[56] P. Martin, R. Nunan, Cellular and molecular mechanisms of repair in acute and chronic wound healing, Br J Dermatol 173(2) (2015) 370-8.

[57] G. Broughton, 2nd, J.E. Janis, C.E. Attinger, The basic science of wound healing, Plast Reconstr Surg 117(7 Suppl) (2006) 12S-34S.

[58] S.A. Eming, M. Hammerschmidt, T. Krieg, A. Roers, Interrelation of immunity and tissue repair or regeneration, Semin Cell Dev Biol 20(5) (2009) 517-27.

[59] S.A. Eming, T. Krieg, J.M. Davidson, Inflammation in wound repair: molecular and cellular mechanisms, The Journal of investigative dermatology 127(3) (2007) 514-25.

[60] P. Maderna, C. Godson, Lipoxins: resolutionary road, Br J Pharmacol 158(4) (2009) 947-59.

[61] M.O. Freire, T.E. Van Dyke, Natural resolution of inflammation, Periodontology 2000 63(1) (2013) 149-64.

[62] C.N. Serhan, S.D. Brain, C.D. Buckley, D.W. Gilroy, C. Haslett, L.A. O'Neill, M. Perretti, A.G. Rossi, J.L. Wallace, Resolution of inflammation: state of the art, definitions and terms, FASEB journal : official publication of the Federation of American Societies for Experimental Biology 21(2) (2007) 325-32.

[63] S.E. Headland, L.V. Norling, The resolution of inflammation: Principles and challenges, Seminars in immunology 27(3) (2015) 149-60.
[64] D. Gilroy, R. De Maeyer, New insights into the resolution of inflammation, Seminars in immunology 27(3) (2015) 161-8.
[65] C.N. Serhan, J. Savill, Resolution of inflammation: the beginning programs the end, Nature immunology 6(12) (2005) 1191-7.
[66] C.N. Serhan, Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways, Annu Rev Immunol 25 (2007) 101-37.
[67] M. Romano, Lipoxin and aspirin-triggered lipoxins, ScientificWorldJournal 10 (2010) 1048-64.
[68] S. Mitchell, G. Thomas, K. Harvey, D. Cottell, K. Reville, G. Berlasconi, N.A. Petasis, L. Erwig, A.J. Rees, J. Savill, H.R. Brady, C. Godson, Lipoxins, aspirin-triggered epi-lipoxins, lipoxin stable analogues, and the resolution of inflammation: stimulation of macrophage phagocytosis of apoptotic neutrophils in vivo, J Am Soc Nephrol 13(10) (2002) 2497-507.
[69] E. Titos, B. Rius, A. Gonzalez-Periz, C. Lopez-Vicario, E. Moran-Salvador, M. Martinez-Clemente, V. Arroyo, J. Claria, Resolvin D1 and its precursor docosahexaenoic acid promote resolution of adipose tissue inflammation by eliciting macrophage polarization toward an M2-like phenotype, Journal of immunology 187(10) (2011) 5408-18.
[70] A. Ariel, C.N. Serhan, Resolvins and protectins in the termination program of acute inflammation, Trends in immunology 28(4) (2007) 176-83.
[71] C.N. Serhan, J. Dalli, R.A. Colas, J.W. Winkler, N. Chiang, Protectins and maresins: New pro-resolving families of mediators in acute inflammation and resolution bioactive metabolome, Biochimica et biophysica acta 1851(4) (2015) 397-413.
[72] C.N. Serhan, R. Yang, K. Martinod, K. Kasuga, P.S. Pillai, T.F. Porter, S.F. Oh, M. Spite, Maresins: novel macrophage mediators with potent antiinflammatory and proresolving actions, J Exp Med 206(1) (2009) 15-23.
[73] C.N. Serhan, Pro-resolving lipid mediators are leads for resolution physiology, Nature 510(7503) (2014) 92-101.
[74] B.N. Brown, S.F. Badylak, Expanded applications, shifting paradigms and an improved understanding of host-biomaterial interactions, Acta biomaterialia 9(2) (2013) 4948-55.
[75] B.D. Ratner, S.J. Bryant, Biomaterials: where we have been and where we are going, Annu Rev Biomed Eng 6 (2004) 41-75.
[76] A. Vishwakarma, N.S. Bhise, M.B. Evangelista, J. Rouwkema, M.R. Dokmeci, A.M. Ghaemmaghami, N.E. Vrana, A. Khademhosseini, Engineering Immunomodulatory

Biomaterials To Tune the Inflammatory Response, Trends Biotechnol 34(6) (2016) 470-82.

[77] S.F. Badylak, TISSUE REGENERATION. A scaffold immune microenvironment, Science 352(6283) (2016) 298.

[78] A.L. Mescher, A.W. Neff, M.W. King, Inflammation and immunity in organ regeneration, Dev Comp Immunol 66 (2017) 98-110.

[79] S.A. Eming, T.A. Wynn, P. Martin, Inflammation and metabolism in tissue repair and regeneration, Science 356(6342) (2017) 1026-1030.

[80] M. Guido, The healing Hand: Man and Wound in the Ancient World Harvard University Press, Cambridge, Massachussets1991.

[81] S. Franz, F. Allenstein, J. Kajahn, I. Forstreuter, V. Hintze, S. Moller, J.C. Simon, Artificial extracellular matrices composed of collagen I and high-sulfated hyaluronan promote phenotypic and functional modulation of human pro-inflammatory M1 macrophages, Acta biomaterialia 9(3) (2013) 5621-9.

[82] Z. Julier, A.J. Park, P.S. Briquez, M.M. Martino, Promoting tissue regeneration by modulating the immune system, Acta biomaterialia 53 (2017) 13-28.

[83] F. Alegre, P. Pelegrin, A.E. Feldstein, Inflammasomes in Liver Fibrosis, Semin Liver Dis 37(2) (2017) 119-127.

[84] N.A. Hotaling, L. Tang, D.J. Irvine, J.E. Babensee, Biomaterial Strategies for Immunomodulation, Annu Rev Biomed Eng 17 (2015) 317-49.

[85] R.M. Gower, R.M. Boehler, S.M. Azarin, C.F. Ricci, J.N. Leonard, L.D. Shea, Modulation of leukocyte infiltration and phenotype in microporous tissue engineering scaffolds via vector induced IL-10 expression, Biomaterials 35(6) (2014) 2024-31.

[86] J.A. Hubbell, S.N. Thomas, M.A. Swartz, Materials engineering for immunomodulation, Nature 462(7272) (2009) 449-60.

[87] T.A. Wynn, A. Chawla, J.W. Pollard, Macrophage biology in development, homeostasis and disease, Nature 496(7446) (2013) 445-55.

[88] D.F. Williams, On the mechanisms of biocompatibility, Biomaterials 29(20) (2008) 2941-53.

[89] A. Mantovani, S.K. Biswas, M.R. Galdiero, A. Sica, M. Locati, Macrophage plasticity and polarization in tissue repair and remodelling, The Journal of pathology 229(2) (2013) 176-85.

[90] J.L. Dziki, L. Huleihel, M.E. Scarritt, S.F. Badylak, Extracellular Matrix Bioscaffolds as Immunomodulatory Biomaterials<sup/>, Tissue Eng Part A 23(19-20) (2017) 1152-1159.

[91] B.N. Brown, R. Londono, S. Tottey, L. Zhang, K.A. Kukla, M.T. Wolf, K.A. Daly, J.E. Reing, S.F. Badylak, Macrophage phenotype as a predictor of constructive remodeling

following the implantation of biologically derived surgical mesh materials, Acta biomaterialia 8(3) (2012) 978-87.

[92] K.L. Spiller, S. Nassiri, C.E. Witherel, R.R. Anfang, J. Ng, K.R. Nakazawa, T. Yu, G. Vunjak-Novakovic, Sequential delivery of immunomodulatory cytokines to facilitate the M1-to-M2 transition of macrophages and enhance vascularization of bone scaffolds, Biomaterials 37 (2015) 194-207.

[93] J. Chen, M. Li, C. Yang, X. Yin, K. Duan, J. Wang, B. Feng, Macrophage phenotype switch by sequential action of immunomodulatory cytokines from hydrogel layers on titania nanotubes, Colloids Surf B Biointerfaces 163 (2018) 336-345.

[94] D.P. Vasconcelos, M. Costa, I.F. Amaral, M.A. Barbosa, A.P. Aguas, J.N. Barbosa, Development of an immunomodulatory biomaterial: Using resolvin D1 to modulate inflammation, Biomaterials 53 (2015) 566-73.

[95] D.P. Vasconcelos, M. Costa, I.F. Amaral, M.A. Barbosa, A.P. Aguas, J.N. Barbosa, Modulation of the inflammatory response to chitosan through M2 macrophage polarization using pro-resolution mediators, Biomaterials 37 (2015) 116-23.

[96] M. Shayan, J. Padmanabhan, A.H. Morris, B. Cheung, R. Smith, J. Schroers, T.R. Kyriakides, Nanopatterned bulk metallic glass-based biomaterials modulate macrophage polarization, Acta biomaterialia 75 (2018) 427-438.

[97] Z. Wang, Y. Cui, J. Wang, X. Yang, Y. Wu, K. Wang, X. Gao, D. Li, Y. Li, X.L. Zheng, Y. Zhu, D. Kong, Q. Zhao, The effect of thick fibers and large pores of electrospun poly(epsilon-caprolactone) vascular grafts on macrophage polarization and arterial regeneration, Biomaterials 35(22) (2014) 5700-10.

[98] C.H. Lee, Y.J. Kim, J.H. Jang, J.W. Park, Modulating macrophage polarization with divalent cations in nanostructured titanium implant surfaces, Nanotechnology 27(8) (2016) 085101.

[99] B. Li, H. Cao, Y. Zhao, M. Cheng, H. Qin, T. Cheng, Y. Hu, X. Zhang, X. Liu, In vitro and in vivo responses of macrophages to magnesium-doped titanium, Sci Rep 7 (2017) 42707.

[100] J.W. Godwin, A.R. Pinto, N.A. Rosenthal, Chasing the recipe for a pro-regenerative immune system, Semin Cell Dev Biol (2016).

[101] M. Lamkanfi, V.M. Dixit, Mechanisms and functions of inflammasomes, Cell 157(5) (2014) 1013-22.

[102] B.K. Davis, H. Wen, J.P. Ting, The inflammasome NLRs in immunity, inflammation, and associated diseases, Annu Rev Immunol 29 (2011) 707-35.

[103] F. Martinon, K. Burns, J. Tschopp, The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta, Molecular cell 10(2) (2002) 417-26.

[104] F. Martinon, A. Mayor, J. Tschopp, The inflammasomes: guardians of the body, Annu Rev Immunol 27 (2009) 229-65.

[105] J. von Moltke, J.S. Ayres, E.M. Kofoed, J. Chavarria-Smith, R.E. Vance, Recognition of bacteria by inflammasomes, Annu Rev Immunol 31 (2013) 73-106.

[106] F. Moghaddas, R. Llamas, D. De Nardo, H. Martinez-Banaclocha, J.J. Martinez-Garcia, P. Mesa-Del-Castillo, P.J. Baker, V. Gargallo, A. Mensa-Vilaro, S. Canna, I.P. Wicks, P. Pelegrin, J.I. Arostegui, S.L. Masters, A novel Pyrin-Associated Autoinflammation with Neutrophilic Dermatosis mutation further defines 14-3-3 binding of pyrin and distinction to Familial Mediterranean Fever, Ann Rheum Dis 76(12) (2017) 2085-2094.

[107] A. Denes, G. Coutts, N. Lenart, S.M. Cruickshank, P. Pelegrin, J. Skinner, N. Rothwell, S.M. Allan, D. Brough, AIM2 and NLRC4 inflammasomes contribute with ASC to acute brain injury independently of NLRP3, Proceedings of the National Academy of Sciences of the United States of America 112(13) (2015) 4050-5.

[108] Y. Qu, S. Misaghi, K. Newton, A. Maltzman, A. Izrael-Tomasevic, D. Arnott, V.M. Dixit, NLRP3 recruitment by NLRC4 during Salmonella infection, J Exp Med 213(6) (2016) 877-85.

[109] W.K. Ip, R. Medzhitov, Macrophages monitor tissue osmolarity and induce inflammatory response through NLRP3 and NLRC4 inflammasome activation, Nat Commun 6 (2015) 6931.

[110] T. Strowig, J. Henao-Mejia, E. Elinav, R. Flavell, Inflammasomes in health and disease, Nature 481(7381) (2012) 278-86.

[111] Y. He, M.Y. Zeng, D. Yang, B. Motro, G. Nunez, NEK7 is an essential mediator of NLRP3 activation downstream of potassium efflux, Nature 530(7590) (2016) 354-7.

[112] H. Shi, Y. Wang, X. Li, X. Zhan, M. Tang, M. Fina, L. Su, D. Pratt, C.H. Bu, S. Hildebrand, S. Lyon, L. Scott, J. Quan, Q. Sun, J. Russell, S. Arnett, P. Jurek, D. Chen, V.V. Kravchenko, J.C. Mathison, E.M. Moresco, N.L. Monson, R.J. Ulevitch, B. Beutler, NLRP3 activation and mitosis are mutually exclusive events coordinated by NEK7, a new inflammasome component, Nature immunology 17(3) (2016) 250-8.

[113] S. Jha, W.J. Brickey, J.P. Ting, Inflammasomes in Myeloid Cells: Warriors Within, Microbiol Spectr 5(1) (2017).

[114] P.J. Baker, D. De Nardo, F. Moghaddas, L.S. Tran, A. Bachem, T. Nguyen, T. Hayman, H. Tye, J.E. Vince, S. Bedoui, R.L. Ferrero, S.L. Masters, Posttranslational Modification as a Critical Determinant of Cytoplasmic Innate Immune Recognition, Physiol Rev 97(3) (2017) 1165-1209.

[115] K. Schroder, J. Tschopp, The inflammasomes, Cell 140(6) (2010) 821-32.

[116] Y. Ogura, F.S. Sutterwala, R.A. Flavell, The inflammasome: first line of the immune response to cell stress, Cell 126(4) (2006) 659-62.

[117] I. Hafner-Bratkovic, P. Pelegrin, Ion homeostasis and ion channels in NLRP3 inflammasome activation and regulation, Curr Opin Immunol 52 (2018) 8-17.

[118] R. Munoz-Planillo, P. Kuffa, G. Martinez-Colon, B.L. Smith, T.M. Rajendiran, G. Nunez, K(+) efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter, Immunity 38(6) (2013) 1142-53.

[119] M. Lamkanfi, V.M. Dixit, Inflammasomes: guardians of cytosolic sanctity, Immunol Rev 227(1) (2009) 95-105.

[120] C. de Torre-Minguela, P. Mesa Del Castillo, P. Pelegrin, The NLRP3 and Pyrin Inflammasomes: Implications in the Pathophysiology of Autoinflammatory Diseases, Frontiers in immunology 8 (2017) 43.

[121] G. dos Santos, M.A. Kutuzov, K.M. Ridge, The inflammasome in lung diseases, Am J Physiol Lung Cell Mol Physiol 303(8) (2012) L627-33.

[122] J. Henao-Mejia, E. Elinav, T. Strowig, R.A. Flavell, Inflammasomes: far beyond inflammation, Nature immunology 13(4) (2012) 321-4.

[123] P. Matzinger, Tolerance, danger, and the extended family, Annu Rev Immunol 12 (1994) 991-1045.

[124] J. Amores-Iniesta, M. Barbera-Cremades, C.M. Martinez, J.A. Pons, B. Revilla-Nuin, L. Martinez-Alarcon, F. Di Virgilio, P. Parrilla, A. Baroja-Mazo, P. Pelegrin, Extracellular ATP Activates the NLRP3 Inflammasome and Is an Early Danger Signal of Skin Allograft Rejection, Cell Rep 21(12) (2017) 3414-3426.

[125] W. Chi, H. Chen, F. Li, Y. Zhu, W. Yin, Y. Zhuo, HMGB1 promotes the activation of NLRP3 and caspase-8 inflammasomes via NF-kappaB pathway in acute glaucoma, J Neuroinflammation 12 (2015) 137.

[126] H.T. Nguyen, K.K. Tran, B. Sun, H. Shen, Activation of inflammasomes by tumor cell death mediated by gold nanoshells, Biomaterials 33(7) (2012) 2197-205.

[127] E.J. Yang, S. Kim, J.S. Kim, I.H. Choi, Inflammasome formation and IL-1beta release by human blood monocytes in response to silver nanoparticles, Biomaterials 33(28) (2012) 6858-67.

[128] C.L. Bueter, C.K. Lee, V.A. Rathinam, G.J. Healy, C.H. Taron, C.A. Specht, S.M. Levitz, Chitosan but not chitin activates the inflammasome by a mechanism dependent upon phagocytosis, The Journal of biological chemistry 286(41) (2011) 35447-55.

[129] A.F. Malik, R. Hoque, X. Ouyang, A. Ghani, E. Hong, K. Khan, L.B. Moore, G. Ng, F. Munro, R.A. Flavell, Y. Shi, T.R. Kyriakides, W.Z. Mehal, Inflammasome components Asc and caspase-1 mediate biomaterial-induced inflammation and foreign body response,

Proceedings of the National Academy of Sciences of the United States of America 108(50) (2011) 20095-100.

[130] A.C. Reisetter, L.V. Stebounova, J. Baltrusaitis, L. Powers, A. Gupta, V.H. Grassian, M.M. Monick, Induction of inflammasome-dependent pyroptosis by carbon black nanoparticles, The Journal of biological chemistry 286(24) (2011) 21844-52.

[131] O. Lunov, T. Syrovets, C. Loos, G.U. Nienhaus, V. Mailander, K. Landfester, M. Rouis, T. Simmet, Amino-functionalized polystyrene nanoparticles activate the NLRP3 inflammasome in human macrophages, ACS nano 5(12) (2011) 9648-57.

[132] D.M. Gomez, S. Urcuqui-Inchima, J.C. Hernandez, Silica nanoparticles induce NLRP3 inflammasome activation in human primary immune cells, Innate Immun 23(8) (2017) 697-708.

[133] M.S. Caicedo, L. Samelko, K. McAllister, J.J. Jacobs, N.J. Hallab, Increasing both CoCrMo-alloy particle size and surface irregularity induces increased macrophage inflammasome activation in vitro potentially through lysosomal destabilization mechanisms, Journal of orthopaedic research : official publication of the Orthopaedic Research Society 31(10) (2013) 1633-42.

[134] C. Nich, Y. Takakubo, J. Pajarinen, M. Ainola, A. Salem, T. Sillat, A.J. Rao, M. Raska, Y. Tamaki, M. Takagi, Y.T. Konttinen, S.B. Goodman, J. Gallo, Macrophages-Key cells in the response to wear debris from joint replacements, Journal of biomedical materials research. Part A 101(10) (2013) 3033-45.

[135] R. Maitra, C.C. Clement, B. Scharf, G.M. Crisi, S. Chitta, D. Paget, P.E. Purdue, N. Cobelli, L. Santambrogio, Endosomal damage and TLR2 mediated inflammasome activation by alkane particles in the generation of aseptic osteolysis, Mol Immunol 47(2-3) (2009) 175-84.

[136] C.A. St Pierre, M. Chan, Y. Iwakura, D.C. Ayers, E.A. Kurt-Jones, R.W. Finberg, Periprosthetic osteolysis: characterizing the innate immune response to titanium wear-particles, Journal of orthopaedic research : official publication of the Orthopaedic Research Society 28(11) (2010) 1418-24.

[137] L. Burton, D. Paget, N.B. Binder, K. Bohnert, B.J. Nestor, T.P. Sculco, L. Santambrogio, F.P. Ross, S.R. Goldring, P.E. Purdue, Orthopedic wear debris mediated inflammatory osteolysis is mediated in part by NALP3 inflammasome activation, Journal of orthopaedic research : official publication of the Orthopaedic Research Society 31(1) (2013) 73-80.

[138] J.I. Andorko, C.M. Jewell, Designing biomaterials with immunomodulatory properties for tissue engineering and regenerative medicine, Bioeng Transl Med 2(2) (2017) 139-155.

[139] D. Fong, P. Gregoire-Gelinas, A.P. Cheng, T. Mezheritsky, M. Lavertu, S. Sato, C.D. Hoemann, Lysosomal rupture induced by structurally distinct chitosans either promotes a

type 1 IFN response or activates the inflammasome in macrophages, Biomaterials 129 (2017) 127-138.

[140] C. Dostert, V. Petrilli, R. Van Bruggen, C. Steele, B.T. Mossman, J. Tschopp, Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica, Science 320(5876) (2008) 674-7.

[141] T. Morishige, Y. Yoshioka, H. Inakura, A. Tanabe, X. Yao, S. Narimatsu, Y. Monobe, T. Imazawa, S. Tsunoda, Y. Tsutsumi, Y. Mukai, N. Okada, S. Nakagawa, The effect of surface modification of amorphous silica particles on NLRP3 inflammasome mediated IL-1beta production, ROS production and endosomal rupture, Biomaterials 31(26) (2010) 6833-42.

[142] V. Hornung, F. Bauernfeind, A. Halle, E.O. Samstad, H. Kono, K.L. Rock, K.A. Fitzgerald, E. Latz, Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization, Nature immunology 9(8) (2008) 847-56.

[143] S.L. Cassel, S.C. Eisenbarth, S.S. Iyer, J.J. Sadler, O.R. Colegio, L.A. Tephly, A.B. Carter, P.B. Rothman, R.A. Flavell, F.S. Sutterwala, The Nalp3 inflammasome is essential for the development of silicosis, Proceedings of the National Academy of Sciences of the United States of America 105(26) (2008) 9035-40.

[144] Y. Lu, S. Xu, H. Chen, M. He, Y. Deng, Z. Cao, H. Pi, C. Chen, M. Li, Q. Ma, P. Gao, Y. Ji, L. Zhang, Z. Yu, Z. Zhou, CdSe/ZnS quantum dots induce hepatocyte pyroptosis and liver inflammation via NLRP3 inflammasome activation, Biomaterials 90 (2016) 27-39.

[145] C. Jin, P. Frayssinet, R. Pelker, D. Cwirka, B. Hu, A. Vignery, S.C. Eisenbarth, R.A. Flavell, NLRP3 inflammasome plays a critical role in the pathogenesis of hydroxyapatite-associated arthropathy, Proceedings of the National Academy of Sciences of the United States of America 108(36) (2011) 14867-72.

[146] S.N. Christo, K.R. Diener, J. Manavis, M.A. Grimbaldeston, A. Bachhuka, K. Vasilev, J.D. Hayball, Inflammasome components ASC and AIM2 modulate the acute phase of biomaterial implant-induced foreign body responses, Sci Rep 6 (2016) 20635.

[147] W.A. Li, B.Y. Lu, L. Gu, Y. Choi, J. Kim, D.J. Mooney, The effect of surface modification of mesoporous silica micro-rod scaffold on immune cell activation and infiltration, Biomaterials 83 (2016) 249-56.

[148] S.C. Eisenbarth, O.R. Colegio, W. O'Connor, F.S. Sutterwala, R.A. Flavell, Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants, Nature 453(7198) (2008) 1122-6.

[149] P. Lagadec, T. Balaguer, F. Boukhechba, G. Michel, S. Bouvet-Gerbettaz, J.M. Bouler, J.C. Scimeca, N. Rochet, Calcium supplementation decreases BCP-induced inflammatory processes in blood cells through the NLRP3 inflammasome down-regulation, Acta biomaterialia 57 (2017) 462-471.

[150] C.L. Bueter, C.K. Lee, J.P. Wang, G.R. Ostroff, C.A. Specht, S.M. Levitz, Spectrum and mechanisms of inflammasome activation by chitosan, Journal of immunology 192(12) (2014) 5943-51.

[151] M. Keller, A. Ruegg, S. Werner, H.D. Beer, Active caspase-1 is a regulator of unconventional protein secretion, Cell 132(5) (2008) 818-31.

[152] E.M. Weinheimer-Haus, R.E. Mirza, T.J. Koh, Nod-like receptor protein-3 inflammasome plays an important role during early stages of wound healing, PloS one 10(3) (2015) e0119106.

[153] H. Ito, A. Kanbe, H. Sakai, M. Seishima, Activation of NLRP3 signalling accelerates skin wound healing, Exp Dermatol 27(1) (2018) 80-86.

[154] T. Ando, H. Ito, A. Kanbe, A. Hara, M. Seishima, Deficiency of NALP3 Signaling Impairs Liver Regeneration After Partial Hepatectomy, Inflammation 40(5) (2017) 1717-1725.

[155] E. Latz, Targeting Inflammasomes in Inflammation, Inflamm Res 59 (2010) S108-S109.

[156] C.M. Artlett, Inflammasomes in wound healing and fibrosis, The Journal of pathology 229(2) (2013) 157-67.

[157] H. He, H. Jiang, Y. Chen, J. Ye, A. Wang, C. Wang, Q. Liu, G. Liang, X. Deng, W. Jiang, R. Zhou, Oridonin is a covalent NLRP3 inhibitor with strong anti-inflammasome activity, Nat Commun 9(1) (2018) 2550.

[158] R.C. Coll, A.A. Robertson, J.J. Chae, S.C. Higgins, R. Munoz-Planillo, M.C. Inserra, I. Vetter, L.S. Dungan, B.G. Monks, A. Stutz, D.E. Croker, M.S. Butler, M. Haneklaus, C.E. Sutton, G. Nunez, E. Latz, D.L. Kastner, K.H. Mills, S.L. Masters, K. Schroder, M.A. Cooper, L.A. O'Neill, A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases, Nature medicine 21(3) (2015) 248-55.

[159] A.G. Baldwin, J. Rivers-Auty, M.J.D. Daniels, C.S. White, C.H. Schwalbe, T. Schilling, H. Hammadi, P. Jaiyong, N.G. Spencer, H. England, N.M. Luheshi, M. Kadirvel, C.B. Lawrence, N.J. Rothwell, M.K. Harte, R.A. Bryce, S.M. Allan, C. Eder, S. Freeman, D. Brough, Boron-Based Inhibitors of the NLRP3 Inflammasome, Cell Chem Biol 24(11) (2017) 1321-1335 e5.

[160] M. Cocco, C. Pellegrini, H. Martinez-Banaclocha, M. Giorgis, E. Marini, A. Costale, G. Miglio, M. Fornai, L. Antonioli, G. Lopez-Castejon, A. Tapia-Abellan, D. Angosto, I. Hafner-Bratkovic, L. Regazzoni, C. Blandizzi, P. Pelegrin, M. Bertinaria, Development of an Acrylate Derivative Targeting the NLRP3 Inflammasome for the Treatment of Inflammatory Bowel Disease, J Med Chem 60(9) (2017) 3656-3671.

[161] H. Jiang, H. He, Y. Chen, W. Huang, J. Cheng, J. Ye, A. Wang, J. Tao, C. Wang, Q. Liu, T. Jin, W. Jiang, X. Deng, R. Zhou, Identification of a selective and direct NLRP3 inhibitor to treat inflammatory disorders, J Exp Med 214(11) (2017) 3219-3238.

[162] E. Latz, The inflammasomes: mechanisms of activation and function, Curr Opin Immunol 22(1) (2010) 28-33.

[163] A. Baroja-Mazo, F. Martin-Sanchez, A.I. Gomez, C.M. Martinez, J. Amores-Iniesta, V. Compan, M. Barbera-Cremades, J. Yague, E. Ruiz-Ortiz, J. Anton, S. Bujan, I. Couillin, D. Brough, J.I. Arostegui, P. Pelegrin, The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response, Nature immunology 15(8) (2014) 738-48.

[164] A. Di Martino, M. Sittinger, M.V. Risbud, Chitosan: a versatile biopolymer for orthopaedic tissue-engineering, Biomaterials 26(30) (2005) 5983-90.

[165] C. Chatelet, O. Damour, A. Domard, Influence of the degree of acetylation on some biological properties of chitosan films, Biomaterials 22(3) (2001) 261-8.

[166] G.J. Blair HS, Law TK, Turkington P., Chitosan and modified chitosan membranes I. Preparation and characterization. , J Appl Polym Sci 33 (1987) 641-56.

[167] K. Tomihata, Y. Ikada, In vitro and in vivo degradation of films of chitin and its deacetylated derivatives, Biomaterials 18(7) (1997) 567-75.

[168] H. Hattori, M. Ishihara, Feasibility of improving platelet-rich plasma therapy by using chitosan with high platelet activation ability, Exp Ther Med 13(3) (2017) 1176-1180.

[169] D.P. Vasconcelos, A.C. Fonseca, M. Costa, I.F. Amaral, M.A. Barbosa, A.P. Aguas, J.N. Barbosa, Macrophage polarization following chitosan implantation, Biomaterials 34(38) (2013) 9952-9.

[170] D. Filion, M. Lavertu, M.D. Buschmann, Ionization and solubility of chitosan solutions related to thermosensitive chitosan/glycerol-phosphate systems, Biomacromolecules 8(10) (2007) 3224-34.

[171] M.D. Buschmann, A. Merzouki, M. Lavertu, M. Thibault, M. Jean, V. Darras, Chitosans for delivery of nucleic acids, Adv Drug Deliv Rev 65(9) (2013) 1234-70.

[172] E.C. Carroll, L. Jin, A. Mori, N. Munoz-Wolf, E. Oleszycka, H.B.T. Moran, S. Mansouri, C.P. McEntee, E. Lambe, E.M. Agger, P. Andersen, C. Cunningham, P. Hertzog, K.A. Fitzgerald, A.G. Bowie, E.C. Lavelle, The Vaccine Adjuvant Chitosan Promotes Cellular Immunity via DNA Sensor cGAS-STING-Dependent Induction of Type I Interferons, Immunity 44(3) (2016) 597-608.

[173] S.V. Madihally, H.W. Matthew, Porous chitosan scaffolds for tissue engineering, Biomaterials 20(12) (1999) 1133-42.

[174] D. Fong, M.B. Ariganello, J. Girard-Lauziere, C.D. Hoemann, Biodegradable chitosan microparticles induce delayed STAT-1 activation and lead to distinct cytokine

responses in differentially polarized human macrophages in vitro, Acta biomaterialia 12 (2015) 183-194.

[175] J. Guzman-Morales, M.B. Ariganello, I. Hammami, M. Thibault, M. Jolicoeur, C.D. Hoemann, Biodegradable chitosan particles induce chemokine release and negligible arginase-1 activity compared to IL-4 in murine bone marrow-derived macrophages, Biochemical and biophysical research communications 405(4) (2011) 538-44.

[176] F. Castro, M.L. Pinto, A.M. Silva, C.L. Pereira, G.Q. Teixeira, M. Gomez-Lazaro, S.G. Santos, M.A. Barbosa, R.M. Goncalves, M.J. Oliveira, Pro-inflammatory chitosan/poly(gamma-glutamic acid) nanoparticles modulate human antigen-presenting cells phenotype and revert their pro-invasive capacity, Acta biomaterialia 63 (2017) 96-109.

[177] M.I. Oliveira, S.G. Santos, M.J. Oliveira, A.L. Torres, M.A. Barbosa, Chitosan drives anti-inflammatory macrophage polarisation and pro-inflammatory dendritic cell stimulation, European cells & materials 24 (2012) 136-52; discussion 152-3.

[178] C.D. Hoemann, G. Chen, C. Marchand, N. Tran-Khanh, M. Thibault, A. Chevrier, J. Sun, M.S. Shive, M.J. Fernandes, P.E. Poubelle, M. Centola, H. El-Gabalawy, Scaffold-guided subchondral bone repair: implication of neutrophils and alternatively activated arginase-1+ macrophages, Am J Sports Med 38(9) (2010) 1845-56.

[179] K. Nishimura, S. Nishimura, N. Nishi, I. Saiki, S. Tokura, I. Azuma, Immunological activity of chitin and its derivatives, Vaccine 2(1) (1984) 93-9.

[180] F. McNab, K. Mayer-Barber, A. Sher, A. Wack, A. O'Garra, Type I interferons in infectious disease, Nature reviews. Immunology 15(2) (2015) 87-103.

[181] S.R. Jameela, A. Misra, A. Jayakrishnan, Cross-linked chitosan microspheres as carriers for prolonged delivery of macromolecular drugs, J Biomater Sci Polym Ed 6(7) (1994) 621-32.

[182] R. Muzzarelli, Baldassarre, V., Conto, F., Ferrara, P., Biagini, G., Gazzanelli, G., & Vasi, V., Biological activity of chitosan: ultrastructural study, Biomaterials 9(3) (1988) 247-252.

[183] Y.J. Seol, J.Y. Lee, Y.J. Park, Y.M. Lee, K. Young, I.C. Rhyu, S.J. Lee, S.B. Han, C.P. Chung, Chitosan sponges as tissue engineering scaffolds for bone formation, Biotechnol Lett 26(13) (2004) 1037-41.

[184] A.R. Costa-Pinto, V.M. Correlo, P.C. Sol, M. Bhattacharya, P. Charbord, B. Delorme, R.L. Reis, N.M. Neves, Osteogenic differentiation of human bone marrow mesenchymal stem cells seeded on melt based chitosan scaffolds for bone tissue engineering applications, Biomacromolecules 10(8) (2009) 2067-73.

[185] A.R. Costa-Pinto, A.J. Salgado, V.M. Correlo, P. Sol, M. Bhattacharya, P. Charbord, R.L. Reis, N.M. Neves, Adhesion, proliferation, and osteogenic differentiation of a mouse

mesenchymal stem cell line (BMC9) seeded on novel melt-based chitosan/polyester 3D porous scaffolds, Tissue Eng Part A 14(6) (2008) 1049-57.

[186] F. Croisier, Jérôme, C., Chitosan-based biomaterials for tissue engineering, European Polymer Journal 49(4) (2013) 780-792.

[187] R. Jayakumar, M. Prabaharan, P.T. Sudheesh Kumar, S.V. Nair, H. Tamura, Biomaterials based on chitin and chitosan in wound dressing applications, Biotechnol Adv 29(3) (2011) 322-37.

[188] W.C. Liu H., Li C., Qin Y., Wang Z., Yang F., Li Z., Wang J., A functional chitosan-based hydrogel as a wound dressing and drug delivery system in the treatment of wound healing, RSC Advances 8 (2018) 7533-7549.

[189] S.K. Nandi, B. Kundu, D. Basu, Protein growth factors loaded highly porous chitosan scaffold: a comparison of bone healing properties, Mater Sci Eng C Mater Biol Appl 33(3) (2013) 1267-75.

[190] Y. Zhou, H.L. Gao, L.L. Shen, Z. Pan, L.B. Mao, T. Wu, J.C. He, D.H. Zou, Z.Y. Zhang, S.H. Yu, Chitosan microspheres with an extracellular matrix-mimicking nanofibrous structure as cell-carrier building blocks for bottom-up cartilage tissue engineering, Nanoscale 8(1) (2016) 309-17.

[191] S.S. Silva, E.G. Popa, M.E. Gomes, M. Cerqueira, A.P. Marques, S.G. Caridade, P. Teixeira, C. Sousa, J.F. Mano, R.L. Reis, An investigation of the potential application of chitosan/aloe-based membranes for regenerative medicine, Acta biomaterialia 9(6) (2013) 6790-7.

[192] C. Intini, L. Elviri, J. Cabral, S. Mros, C. Bergonzi, A. Bianchera, L. Flammini, P. Govoni, E. Barocelli, R. Bettini, M. McConnell, 3D-printed chitosan-based scaffolds: An in vitro study of human skin cell growth and an in-vivo wound healing evaluation in experimental diabetes in rats, Carbohydr Polym 199 (2018) 593-602.

[193] Y. Shu, Y. Yu, S. Zhang, J. Wang, Y. Xiao, C. Liu, The immunomodulatory role of sulfated chitosan in BMP-2-mediated bone regeneration, Biomater Sci 6(9) (2018) 2496-2507.

[194] V. Campani, E. Pagnozzi, I. Mataro, L. Mayol, A. Perna, F. D'Urso, A. Carillo, M. Cammarota, M.C. Maiuri, G. De Rosa, Chitosan Gel to Treat Pressure Ulcers: A Clinical Pilot Study, Pharmaceutics 10(1) (2018).

[195] S.H. Kang, D. Han, S. Kim, C.H. Yoon, J.J. Park, J.W. Suh, Y.S. Cho, T.J. Youn, I.H. Chae, Hemostasis pad combined with compression device after transradial coronary procedures: A randomized controlled trial, PloS one 12(7) (2017) e0181099.

[196] A.G. Robling, A.B. Castillo, C.H. Turner, Biomechanical and molecular regulation of bone remodeling, Annu Rev Biomed Eng 8 (2006) 455-98.

[197] B. Clarke, Normal bone anatomy and physiology, Clin J Am Soc Nephrol 3 Suppl 3 (2008) S131-9.

[198] A.L. Boskey, Bone composition: relationship to bone fragility and antiosteoporotic drug effects, Bonekey Rep 2 (2013) 447.

[199] R.J. Miron, D.D. Bosshardt, OsteoMacs: Key players around bone biomaterials, Biomaterials 82 (2016) 1-19.

[200] S.L. Teitelbaum, F.P. Ross, Genetic regulation of osteoclast development and function, Nat Rev Genet 4(8) (2003) 638-49.

[201] W.J. Boyle, W.S. Simonet, D.L. Lacey, Osteoclast differentiation and activation, Nature 423(6937) (2003) 337-42.

[202] H.C. Blair, N.A. Athanasou, Recent advances in osteoclast biology and pathological bone resorption, Histol Histopathol 19(1) (2004) 189-99.

[203] A.M.M. MARK F. PITTENGER, STEPHEN C. BECK, RAMA K. JAISWAL, ROBIN DOUGLAS, JOSEPH D. MOSCA, MARK A. MOORMAN, DONALD W. SIMONETTI, STEWART CRAIG, DANIEL R. MARSHAK, Multilineage Potential of Adult Human Mesenchymal Stem Cells, Science 284(5411) (1999) 143-147.

[204] S.W. Kim, P.D. Pajevic, M. Selig, K.J. Barry, J.Y. Yang, C.S. Shin, W.Y. Baek, J.E. Kim, H.M. Kronenberg, Intermittent parathyroid hormone administration converts quiescent lining cells to active osteoblasts, J Bone Miner Res 27(10) (2012) 2075-84.

[205] R.T. Turner, U.T. Iwaniec, C.P. Wong, L.B. Lindenmaier, L.A. Wagner, A.J. Branscum, S.A. Menn, J. Taylor, Y. Zhang, H. Wu, J.D. Sibonga, Acute exposure to high dose gamma-radiation results in transient activation of bone lining cells, Bone 57(1) (2013) 164-73.

[206] I. Matic, B.G. Matthews, X. Wang, N.A. Dyment, D.L. Worthley, D.W. Rowe, D. Grcevic, I. Kalajzic, Quiescent Bone Lining Cells Are a Major Source of Osteoblasts During Adulthood, Stem Cells 34(12) (2016) 2930-2942.

[207] V. Everts, J.M. Delaisse, W. Korper, D.C. Jansen, W. Tigchelaar-Gutter, P. Saftig, W. Beertsen, The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation, J Bone Miner Res 17(1) (2002) 77-90.

[208] L.F. Bonewald, Establishment and characterization of an osteocyte-like cell line, MLO-Y4, J Bone Miner Metab 17(1) (1999) 61-5.

[209] L. Xing, B.F. Boyce, Regulation of apoptosis in osteoclasts and osteoblastic cells, Biochemical and biophysical research communications 328(3) (2005) 709-20.

[210] M.K. Chang, L.J. Raggatt, K.A. Alexander, J.S. Kuliwaba, N.L. Fazzalari, K. Schroder, E.R. Maylin, V.M. Ripoll, D.A. Hume, A.R. Pettit, Osteal tissue macrophages are intercalated throughout human and mouse bone lining tissues and regulate osteoblast function in vitro and in vivo, Journal of immunology 181(2) (2008) 1232-44.

[211] K.A. Alexander, M.K. Chang, E.R. Maylin, T. Kohler, R. Muller, A.C. Wu, N. Van Rooijen, M.J. Sweet, D.A. Hume, L.J. Raggatt, A.R. Pettit, Osteal macrophages promote in vivo intramembranous bone healing in a mouse tibial injury model, J Bone Miner Res 26(7) (2011) 1517-32.

[212] S.W. Cho, F.N. Soki, A.J. Koh, M.R. Eber, P. Entezami, S.I. Park, N. van Rooijen, L.K. McCauley, Osteal macrophages support physiologic skeletal remodeling and anabolic actions of parathyroid hormone in bone, Proceedings of the National Academy of Sciences of the United States of America 111(4) (2014) 1545-50.

[213] I.G. Winkler, N.A. Sims, A.R. Pettit, V. Barbier, B. Nowlan, F. Helwani, I.J. Poulton, N. van Rooijen, K.A. Alexander, L.J. Raggatt, J.P. Levesque, Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs, Blood 116(23) (2010) 4815-28.

[214] A.D. Berendsen, B.R. Olsen, Bone development, Bone 80 (2015) 14-18.

[215] L.F. Bonewald, The amazing osteocyte, J Bone Miner Res 26(2) (2011) 229-38.

[216] H.K. Datta, W.F. Ng, J.A. Walker, S.P. Tuck, S.S. Varanasi, The cell biology of bone metabolism, J Clin Pathol 61(5) (2008) 577-87.

[217] E.M. Hauge, D. Qvesel, E.F. Eriksen, L. Mosekilde, F. Melsen, Cancellous bone remodeling occurs in specialized compartments lined by cells expressing osteoblastic markers, J Bone Miner Res 16(9) (2001) 1575-82.

[218] D. Lopes, C. Martins-Cruz, M.B. Oliveira, J.F. Mano, Bone physiology as inspiration for tissue regenerative therapies, Biomaterials 185 (2018) 240-275.

[219] T.A. Einhorn, L.C. Gerstenfeld, Fracture healing: mechanisms and interventions, Nat Rev Rheumatol 11(1) (2015) 45-54.

[220] F. Loi, L.A. Cordova, J. Pajarinen, T.H. Lin, Z. Yao, S.B. Goodman, Inflammation, fracture and bone repair, Bone 86 (2016) 119-30.

[221] L.C. Gerstenfeld, Y.M. Alkhiary, E.A. Krall, F.H. Nicholls, S.N. Stapleton, J.L. Fitch, M. Bauer, R. Kayal, D.T. Graves, K.J. Jepsen, T.A. Einhorn, Three-dimensional reconstruction of fracture callus morphogenesis, J Histochem Cytochem 54(11) (2006) 1215-28.

[222] J.J. El-Jawhari, E. Jones, P.V. Giannoudis, The roles of immune cells in bone healing; what we know, do not know and future perspectives, Injury 47(11) (2016) 2399-2406.

[223] G.S. Baht, L. Vi, B.A. Alman, The Role of the Immune Cells in Fracture Healing, Curr Osteoporos Rep 16(2) (2018) 138-145.

[224] F. Shapiro, Bone development and its relation to fracture repair. The role of mesenchymal osteoblasts and surface osteoblasts, European cells & materials 15 (2008) 53-76.

[225] R. Marsell, T.A. Einhorn, The biology of fracture healing, Injury 42(6) (2011) 551-5.
[226] P.V. Giannoudis, T.A. Einhorn, D. Marsh, Fracture healing: the diamond concept, Injury 38 Suppl 4 (2007) S3-6.
[227] J.G. Andrew, S.M. Andrew, A.J. Freemont, D.R. Marsh, Inflammatory cells in normal human fracture healing, Acta Orthop Scand 65(4) (1994) 462-6.
[228] L. Gao, D. Faibish, G. Fredman, B.S. Herrera, N. Chiang, C.N. Serhan, T.E. Van Dyke, R. Gyurko, Resolvin E1 and chemokine-like receptor 1 mediate bone preservation, Journal of immunology 190(2) (2013) 689-94.
[229] T. Kon, T.J. Cho, T. Aizawa, M. Yamazaki, N. Nooh, D. Graves, L.C. Gerstenfeld, T.A. Einhorn, Expression of osteoprotegerin, receptor activator of NF-kappaB ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing, J Bone Miner Res 16(6) (2001) 1004-14.
[230] M.N. Knight, K.D. Hankenson, Mesenchymal Stem Cells in Bone Regeneration, Adv Wound Care (New Rochelle) 2(6) (2013) 306-316.
[231] M.V. Thomas, D.A. Puleo, Infection, inflammation, and bone regeneration: a paradoxical relationship, J Dent Res 90(9) (2011) 1052-61.
[232] J.A. Cameron, D.J. Milner, J.S. Lee, J. Cheng, N.X. Fang, I.M. Jasiuk, Employing the biology of successful fracture repair to heal critical size bone defects, Curr Top Microbiol Immunol 367 (2013) 113-32.
[233] R. Dimitriou, E. Tsiridis, P.V. Giannoudis, Current concepts of molecular aspects of bone healing, Injury 36(12) (2005) 1392-404.
[234] B. McKibbin, The biology of fracture healing in long bones, J Bone Joint Surg Br 60-B(2) (1978) 150-62.
[235] A. Schindeler, M.M. McDonald, P. Bokko, D.G. Little, Bone remodeling during fracture repair: The cellular picture, Semin Cell Dev Biol 19(5) (2008) 459-66.
[236] B. Strömqvist, Hip fracture in rheumatoid aethritis, Acta Orthop Scand 55 (1984) 624-628.
[237] R.A. Kayal, D. Tsatsas, M.A. Bauer, B. Allen, M.O. Al-Sebaei, S. Kakar, C.W. Leone, E.F. Morgan, L.C. Gerstenfeld, T.A. Einhorn, D.T. Graves, Diminished bone formation during diabetic fracture healing is related to the premature resorption of cartilage associated with increased osteoclast activity, J Bone Miner Res 22(4) (2007) 560-8.
[238] B. Dominiak, W. Oxberry, P. Chen, Study on a nonhealing fracture from a patient with systemic lupus erythematosus and its pathogenetic mechanisms, Ultrastruct Pathol 29(2) (2005) 107-20.
[239] L. Claes, S. Recknagel, A. Ignatius, Fracture healing under healthy and inflammatory conditions, Nat Rev Rheumatol 8(3) (2012) 133-43.

[240] R. Hardy, M.S. Cooper, Bone loss in inflammatory disorders, J Endocrinol 201(3) (2009) 309-20.

[241] J.A. Clowes, B.L. Riggs, S. Khosla, The role of the immune system in the pathophysiology of osteoporosis, Immunol Rev 208 (2005) 207-27.

[242] R.A. Kayal, M. Siqueira, J. Alblowi, J. McLean, N. Krothapalli, D. Faibish, T.A. Einhorn, L.C. Gerstenfeld, D.T. Graves, TNF-alpha mediates diabetes-enhanced chondrocyte apoptosis during fracture healing and stimulates chondrocyte apoptosis through FOXO1, J Bone Miner Res 25(7) (2010) 1604-15.

[243] L. Batoon, S.M. Millard, M.E. Wullschleger, C. Preda, A.C. Wu, S. Kaur, H.W. Tseng, D.A. Hume, J.P. Levesque, L.J. Raggatt, A.R. Pettit, CD169(+) macrophages are critical for osteoblast maintenance and promote intramembranous and endochondral ossification during bone repair, Biomaterials (2017).

[244] M. Ishikawa, H. Ito, T. Kitaori, K. Murata, H. Shibuya, M. Furu, H. Yoshitomi, T. Fujii, K. Yamamoto, S. Matsuda, MCP/CCR2 signaling is essential for recruitment of mesenchymal progenitor cells during the early phase of fracture healing, PloS one 9(8) (2014) e104954.

[245] C.M. Champagne, J. Takebe, S. Offenbacher, L.F. Cooper, Macrophage cell lines produce osteoinductive signals that include bone morphogenetic protein-2, Bone 30(1) (2002) 26-31.

[246] L. Vi, G.S. Baht, H. Whetstone, A. Ng, Q. Wei, R. Poon, S. Mylvaganam, M. Grynpas, B.A. Alman, Macrophages promote osteoblastic differentiation in-vivo: implications in fracture repair and bone homeostasis, J Bone Miner Res 30(6) (2015) 1090-102.

[247] V. Nicolaidou, M.M. Wong, A.N. Redpath, A. Ersek, D.F. Baban, L.M. Williams, A.P. Cope, N.J. Horwood, Monocytes induce STAT3 activation in human mesenchymal stem cells to promote osteoblast formation, PloS one 7(7) (2012) e39871.

[248] A. Ho-Shui-Ling, J. Bolander, L.E. Rustom, A.W. Johnson, F.P. Luyten, C. Picart, Bone regeneration strategies: Engineered scaffolds, bioactive molecules and stem cells current stage and future perspectives, Biomaterials 180 (2018) 143-162.

[249] L. Parisi, A. Toffoli, G. Ghiacci, G.M. Macaluso, Tailoring the Interface of Biomaterials to Design Effective Scaffolds, J Funct Biomater 9(3) (2018).

[250] K.L. Spiller, G. Vunjak-Novakovic, Clinical translation of controlled protein delivery systems for tissue engineering, Drug Deliv Transl Res 5(2) (2015) 101-15.

[251] M.M.-O. Wernike E., Liu Y., Wismeijei D., Hunziker E.B., Siebenrock K.-A., Hofstetter W., Klenke, F.M. , Vegf incorporated into calcium phosphate ceramics promotes vascularisation and bone formation in vivo, European Cells and Materials 19 (2010) 30-40.

[252] H.T. Aro, S. Govender, A.D. Patel, P. Hernigou, A. Perera de Gregorio, G.I. Popescu, J.D. Golden, J. Christensen, A. Valentin, Recombinant human bone morphogenetic protein-2: a randomized trial in open tibial fractures treated with reamed nail fixation, J Bone Joint Surg Am 93(9) (2011) 801-8.

[253] M. Shi, Z. Chen, S. Farnaghi, T. Friis, X. Mao, Y. Xiao, C. Wu, Copper-doped mesoporous silica nanospheres, a promising immunomodulatory agent for inducing osteogenesis, Acta biomaterialia 30 (2016) 334-344.

[254] R. Mirza, T.J. Koh, Dysregulation of monocyte/macrophage phenotype in wounds of diabetic mice, Cytokine 56(2) (2011) 256-64.

[255] K.A. Kigerl, J.C. Gensel, D.P. Ankeny, J.K. Alexander, D.J. Donnelly, P.G. Popovich, Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord, J Neurosci 29(43) (2009) 13435-44.

[256] K.L. Spiller, R.R. Anfang, K.J. Spiller, J. Ng, K.R. Nakazawa, J.W. Daulton, G. Vunjak-Novakovic, The role of macrophage phenotype in vascularization of tissue engineering scaffolds, Biomaterials 35(15) (2014) 4477-88.

[257] J.R. Alhamdi, T. Peng, I.M. Al-Naggar, K.L. Hawley, K.L. Spiller, L.T. Kuhn, Controlled M1-to-M2 transition of aged macrophages by calcium phosphate coatings, Biomaterials (2018).

[258] A.S. Antonopoulos, M. Margaritis, R. Lee, K. Channon, C. Antoniades, Statins as anti-inflammatory agents in atherogenesis: molecular mechanisms and lessons from the recent clinical trials, Curr Pharm Des 18(11) (2012) 1519-30.

[259] T. Li, M. Peng, Z. Yang, X. Zhou, Y. Deng, C. Jiang, M. Xiao, J. Wang, 3D-printed IFN-gamma-loading calcium silicate-beta-tricalcium phosphate scaffold sequentially activates M1 and M2 polarization of macrophages to promote vascularization of tissue engineering bone, Acta biomaterialia 71 (2018) 96-107.

[260] Z.W. Zheng, Y.H. Chen, D.Y. Wu, J.B. Wang, M.M. Lv, X.S. Wang, J. Sun, Z.Y. Zhang, Development of an Accurate and Proactive Immunomodulatory Strategy to Improve Bone Substitute Material-Mediated Osteogenesis and Angiogenesis, Theranostics 8(19) (2018) 5482-5500.

[261] E. Gomez-Barrena, P. Rosset, D. Lozano, J. Stanovici, C. Ermthaller, F. Gerbhard, Bone fracture healing: cell therapy in delayed unions and nonunions, Bone 70 (2015) 93-101.

[262] P. Matzinger, T. Kamala, Tissue-based class control: the other side of tolerance, Nature reviews. Immunology 11(3) (2011) 221-30.

[263] https://www.globaldata.com, Global orthopedics market. 2018).

[264] B.N. Summers, S.M. Eisenstein, Donor site pain from the ilium. A complication of lumbar spine fusion, J Bone Joint Surg Br 71(4) (1989) 677-80.
[265] P.V. Giannoudis, H. Dinopoulos, E. Tsiridis, Bone substitutes: an update, Injury 36 Suppl 3 (2005) S20-7.

# CHAPTER II

## AIMS & MOTIVATIONS

# AIMS & MOTIVATIONS

Designing materials to modulate the immune response requires an understanding of the role of the immune system in physiologic processes including tissue repair, development and tissue homeostasis.

Chitosan-based products have several interesting properties that could be useful on the tissue engineering field. A remarkable one is the capacity to induce a pro- or an anti-inflammatory response depending on their physical and chemical properties.

The immune response is fundamental for tissue repair, and Ch scaffolds can be used as a template for new tissue formation. The implantation of a biomaterial causes injury to the tissue and leads to the onset of an immune response. It begins with an innate response, which includes macrophages and the release of several immune factors, that will trigger a cascade of immune responders. So, it is important to deeply understand macrophage behavior towards a specific biomaterial. The development of materials able to modulate macrophage responses appears as a new trend in regenerative medicine.

The NLRP3 inflammasome, one of the biggest intracellular complexes activated during an immune response to several danger signals, was described to have an important role in the inflammatory response to Ch-based nanoproducts. However, due to the lack of studies showing the role of macroscopic Ch products on NLRP3 inflammasome activation, we decided in this research work to understand: (i) the role of a recently described intracellular pathway in the macrophage response to macroscopic Ch-based products, the NLRP3 inflammasome pathway; (ii) develop an immunomodulatory material, through the sequential transition of macrophage polarization from a M1 to a M2 macrophage phenotype and finally (iii) verify in an *in vivo* model of bone tissue repair the capacity of the developed material to improve bone healing.

**Hypothesis and goal**

Throughout the development of this research work I have explored the idea that Ch-based products have favorable immunomodulatory properties that can shift the default response to a foreign body implant towards one of tissue integration and functional remodeling.

In order to test this hypothesis, experiments were conducted using a 3D Ch scaffold with different DA.

*In vitro* studies were performed using primary mouse and human macrophages and the *in vivo* studies using two different animal models: a mouse model of inflammation and a rat femoral defect model (tissue injury model).

**Specific Aims**

**i.** Investigate the NLRP3 inflammasome role in the inflammatory response to 3D Ch scaffolds with different DA. To accomplish this goal, we analyzed the inflammasome activation in primary and human macrophages cultured in 3D Ch scaffolds with different DA. Cytokine production, cell death and NLRP3 oligomerization were evaluated. Chitosan scaffolds were not able to activate *per se* NLRP3 inflammasome. Interestingly Ch scaffolds revealed to have an immunomodulatory effect in the NLRP3 inflammasome activation. These results are presented in Chapter III.

**ii.** Development of a strategy to deliver a pro-resolving lipid mediator to modulate macrophage response to 3D Ch scaffolds. In order to achieve this objective, a pro-resolving mediator, Resolvin D1 was incorporated in 3D Ch scaffolds with a higher DA (described to induce a M1 macrophage phenotype). The developed material was implanted in an *in vivo* model of inflammation, the air pouch model. The inflammatory exudates, the cells that were recruited to the implant site and Ch scaffolds were analyzed. The results showed that the developed material was able to modulate the macrophage response to 3D Ch scaffolds, leading to an increase of M2/M1 macrophage ratio. These results are described in Chapter IV.

**iii.** Evaluate the effect of the material developed in (ii) in an *in vivo* model of bone injury. To test the developed material rat femoral defect model was used. Animals were evaluated two months post-injury for the new bone formation and Coll deposition. The femurs were collected and analyzed by μCT and histological analysis. We observed an improvement in trabecular thickness and in Coll type I in the animals that received the material developed in (ii). These results are described in Chapter V.

# CHAPTER III

## ORIGINAL RESEARCH WORK

### "THE ROLE OF NLRP3 INFLAMMASOME IN THE INFLAMMATORY RESPONSE TO MACROSCOPIC BIOMATERIALS"

**ARTICLE I**

"3D chitosan scaffolds impair NLRP3 inflammasome response in macrophages." (2019). Acta Biomaterialia.

**3D Chitosan scaffolds impair NLRP3 inflammasome response in macrophages**

Daniela P. Vasconcelos[1,2,3], Carlos de Torre-Minguela[5], Ana I. Gomez[5], Artur P. Águas[3,4], Mário A. Barbosa[1,2,3], Pablo Pelegrín[5#§] and Judite N. Barbosa[1,2,3#*]

1 - i3S - Instituto de Inovação e Investigação em Saúde, Universidade do Porto, Rua Alfredo Allen, 208, 4200-125 Porto, Portugal.

2 - INEB - Instituto de Engenharia Biomédica, Rua Alfredo Allen, 208, 4200-125 Porto, Portugal.

3 - ICBAS - Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal.

4 - UMIB - Unit for Multidisciplinary Biomedical Research of ICBAS - Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal.

5 - Molecular Inflammation Group, Biomedical Research Institute of Murcia (IMIB-Arrixaca), Hospital Clínico Universitario Virgen de la Arrixaca, Carretera Buenavista, 30120 Murcia, Spain.

[#] Shared last authorship

## *ABSTRACT*

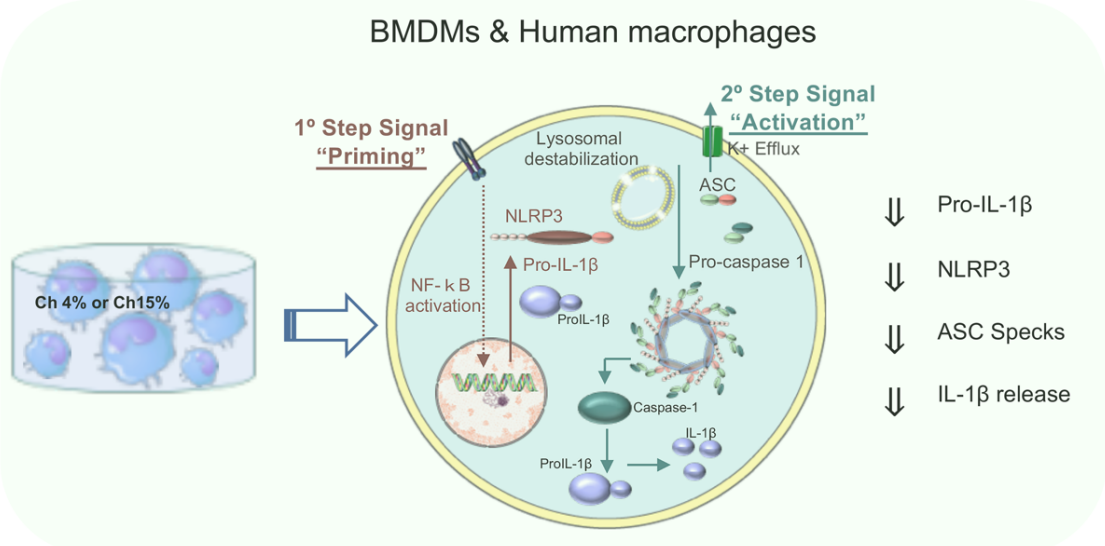
Chitosan (Ch) is used in different biomedical applications to promote tissue repair. However, tissue injury caused by biomaterial implantation lead to the release of danger signals that engage different inflammatory pathways on the host. Different implanted materials activate the inflammasome leading to the modulation of the immune response. Here we have studied how macroscopic biomaterials, Ch scaffolds with different chemical composition: 4% or 15% degree of acetylation (DA) modulate the activation of the NLRP3 inflammasome in vitro. For that, we assessed the NLRP3 inflammasome in bone marrow derived mouse macrophages (BMDM) and human macrophages cultured within 3D Ch scaffolds. We found that both Ch scaffolds did not trigger the NLRP3 inflammasome activation in macrophages. Furthermore, BMDMs and human macrophages cultured in both Ch scaffolds presented a reduction in the number of apoptosis-associated speck-like protein containing a caspase activating recruitment domain (ASC) specks and in IL-1β release upon classical NLRP3 inflammasome stimulation. We also found a decrease in proIL-1β in BMDMs after priming with LPS when cultured in Ch scaffolds with DA 4% DA after priming with LPS when compared to Ch scaffolds with 15% DA or to macrophages cultured in cell-culture plates. Our results shows that 3D Ch scaffolds with different DA impair NLRP3 inflammasome priming and activation.



## *KEYWORDS*

Biomaterials; macrophages; inflammasomes; interleukin-1; immunomodulation; 3D scaffolds


## *GRAPHICAL ABSTRACT*



## *1. INTRODUCTION*

Chitosan (Ch) is a glucosamine and N-acetyl glucosamine polysaccharide widely used in biomedical applications to achieve tissue repair through mechanisms involving biological responses that are still poorly understood [1-6]. The immune response is intimately involved in the success of tissue repair or its potential dysregulations [7, 8]. Tissue repair is divided in three stages that could partly occur at the same time: inflammation, new tissue formation, and remodeling [8, 9]. Due to their plasticity, macrophages have an important role in all of the three stages [10]. In recent years, the immune-mediated tissue repair triggered by a biomaterial has become an emergent strategy to repair tissue damage [11, 12]. Different approaches can be used to obtain a desired immune response, most of them through the modulation of macrophage functions [11, 13-19]. Although several studies have demonstrated the immunomodulatory properties of Ch, the exact intracellular pathways that are involved in these events have started to be elucidated [13, 20-22]. Recently, the nucleotide-binding oligomerization domain-like receptor containing pyrin domain 3 (NLRP3) inflammasome and cGAS – STING (cyclic GMP-AMP synthase-stimulator of interferon genes) were identified as signal pathways involved in the cellular immune response to Ch products [23-25].

Tissue injury after biomaterial implantation leads to the release of danger signals that are responsible in part for an inflammatory response that, if it is exacerbated and continued during the time it may impair the function of the implant [26]. Danger signals, such as alarmins, released from injured tissues are associated with the activation of the inflammasome. Moreover, inflammasome components were recently found to modulate foreign body response to implanted materials [27]. The inflammasomes are immune platforms that tightly regulate the production of the pro-inflammatory cytokines interleukin (IL)-1$\beta$ and IL-18 [28, 29]. Several studies have indicated that Ch is a powerful activator of the NLRP3 inflammasome, being the phagocytosis of Ch particles pivotal for this activation [24, 25, 30]. The NLRP3 inflammasome is a major component of the innate immune system and it is expressed mainly by myeloid cells. NLRP3 is upregulated in response to different pathogen-associated molecule patterns (PAMPs), or danger-associated molecule patterns (DAMPs) that are linked to tissue injury or tissue stress [31-33]. The NLRP3 inflammasome is formed by a cytosolic sensor NOD-Like receptor (NLR) that is the NLRP3 protein, an adaptor protein called apoptosis-associated speck-like protein containing a caspase activating recruitment domain (ASC), and the cysteine protease pro-caspase-1 as the effector molecule. The activation of the NLRP3 inflammasome is closely regulated by two-

step signals in macrophages [34]. The first “priming” signal enhances the expression of the inflammasome components and target proteins via activation of the nuclear factor (NF)-κB transcription factor [35]. This first signal also result in a post-transcriptional priming of the NLRP3 protein mainly involving deubiquitination [36, 37]. The second “activation” signal promotes the assembly of the inflammasome components, and pro-caspase-1 undergoes autocatalytic processing resulting in caspase-1 activation which in turn cleaves the pro-IL-1β and pro-IL-18 into the mature and active forms. Caspase-1 also cleaves gasdermin D to promote plasma membrane pore formation, the release of the mature cytokines and pyroptotic cell death [38, 39]. The second NLRP3 activating signal involves three major mechanisms, including generation of reactive oxygen species (ROS), lysosomal damage, and intracellular potassium efflux [40-42]. Inflammasome activation by biomaterials has been explored mostly with engineered nanomaterials [43, 44].

Studies that show inflammasome activation by Ch are related to nanoscale Ch products and Ch activates NLRP3 by a mechanism that is dependent on phagocytosis [24] and lysosomal destabilization [25]. In contrast, the activation of inflammasome by large scale Ch structures has not been investigated yet. Tissue engineering approaches, such as for example those for bone repair comprises the implantation of macroscopic scaffolds or devices [45]. Thus, advanced information on how these large Ch materials modulate the NLRP3 inflammasome will be of key importance to improve biomaterial integration. In this study, we have investigated the activation of the NLRP3 inflammasome in macrophages cultured *in vitro* into 3D Ch scaffolds with different degree of acetylation (DA). For that, we have used both primary mouse BMDMs and human macrophages derived from primary blood monocytes of healthy donors. We found that 3D Ch scaffolds inhibit the activation of the NLRP3 inflammasome, by affecting both the priming and the activation step. To our knowledge this is the first work that demonstrates the modulatory capacity of 3D porous Ch scaffolds in the activation of the NLRP3 inflammasome.

## 2. MATERIALS & METHODS

### 2.1. Chitosan 3D scaffolds preparation

Squid pen Ch (ref. 114, Batch No. S4; DA w2%; Mahtani Chitosan Pvt. Ltd) was purified through filtration of Ch acidic solution and subsequent alkali precipitation. The DA of the purified Ch was assessed by Fourier transform infrared spectroscopy (FTIR) and a DA of 4% was obtained. This Ch was subsequently N-acetylated in order to prepare Ch with a DA of 15% according to the method described by Vachoud *et al*. [46], in a water/acetic acid/1,2-propanediol solution, using acetic anhydride as reactive. The 3D porous scaffolds were prepared from both Ch solutions (4 and 15%) through thermally induced phase separation (-20°C) and consequent sublimation of the ice crystals. Following lyophilization (-85°C; 0.2 mbar; 24h), the obtained scaffolds were prepared as discs having a diameter of 4 mm (for BMDM experiments) and 11mm (for human macrophages experiments) and a thickness of 2mm. In accordance to previous results of ours, these scaffolds revealed for both of the DA that were used, a highly porous and homogeneous microstructure with interconnected pores with diameters in the range of 100 μm [47].

### 2.2. Mouse bone marrow-derived macrophages (BMDM)

C57BL/6 (wild type, WT) mice were purchased from Harlan. ASC-deficient (*Pycard* $^{-/-}$) [48] and Caspase-1/11 deficient (*Casp1/11*$^{-/-}$) [49] mice were in C57BL/6 background. For all experiments, mice between 8-10 weeks of age bred under SPF conditions were used and in accordance with the Spanish national (RD 1201/2005 and Law 32/2007) and EU (86/609/EEC and 2010/63/EU) legislation, no specific approval is needed to extract tissues from euthanized mice. BMDM were obtained from femurs and tibias of mice euthanized by $CO_2$ inhalation as previously described [50]. Briefly, the bone marrow was flushed out and resuspended in DMEM (LONZA, Verviers, Belgium) supplemented with 25% of L929 medium containing macrophage colony-stimulating factor, 15% FCS (Invitrogen Life Technologies, Madrid, Spain), 100U/ml penicillin-streptomycin (Lonza), and 1% L-glutamine (Lonza) and plated onto 150-mm dishes, and cultures at 37°C with 5% $CO_2$. After 6 days, the resulting BMDM were detached with cold PBS, and seeded into the Ch scaffolds at a confluence of 0.328 x $10^6$ cells/cm$^2$, left to adhere for 3h at 37°C with 5% $CO_2$ and primed at day 7 as described below. As controls, the same number of cells were plated in wells without Ch scaffold. The macrophage purity of these preparations was > 90% F4/80 positive cells. Cells were primed for 4h at 37°C with 1 μg/ml of *Escherichia coli* LPS serotype 055:B5 (Sigma – Aldrich), rinsed three times with Et-buffer (147 mM NaCl, 10 mM HEPES, 13 mM D-glucose, 2 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$; pH 7.4) and incubated in the

same buffer at 37°C with 10 μM of nigericin (Sigma – Aldrich) during 20 min for NLRP3 activation.

### *2.3. Human monocytes and monocyte-derived macrophages*

Human monocytes were isolated by negative selection from healthy donors buffy coats by using RosetteSep human monocyte enrichment cocktail (StemCell Technologies), as previously described [51, 52]. The buffy coats were provided by the Immunohemotherapy Department of Centro Hospitalar São João (CHSJ) from Porto (Portugal) with the approval of the clinical ethical committee (Protocol reference 260/11). Cells were resuspended in complete medium (RPMI 1640 medium (Corning) supplemented with 10% FBS (Biowest), 100 U/mL penicillin, 100 μg/mL streptomycin (both from Immunotools). For macrophage differentiation, cells were plated in Ch scaffolds at a confluence of $0.5 \times 10^6$ monocytes/scaffold and left to adhere for 3 h. As controls, the same number of cells were plated in tissue culture plates (TCPS). Monocytes were cultured for 6 days in complete medium and 50 ng/ml of macrophage colony-stimulating factor (M-CSF) (Immunotools). After 6 days of differentiation the medium was changed, cells were washed and fresh medium without M-CSF was added. At day 7, cells were LPS-primed and stimulated with nigericin using the same conditions used for the BMDM. Controls were left unstimulated.

### *2.4. Lactate dehydrogenase (LDH)*

The presence of LDH in cell-free supernatants was measured using the cytotoxicity detection kit (Roche, Barcelona, Spain) following the manufacturer's instructions, and read in a synergy Mx plate reader (BioTek). Lactate dehydrogenase was used as marker for cell death. Cell death was defined as the percentage of released LDH compared with maximal LDH activity from cell lysates obtained using 1% Triton X-100 on cells plated on TCPS.

### *2.5. ELISA assays*

Different cytokines were quantified in cell culture supernatants using commercially available ELISA kits. Mouse and human IL-1β (Mouse -Ready-SET-Go, eBiosciene; Human – Quantikine, R&D), were performed according to the supplier indications.

### *2.6. Immunocytochemistry and confocal microscopy*

Macrophages stimulated on coverslips and Ch scaffolds were fixed for 15 min with 4% formaldehyde, and then washed three times with PBS. For ASC speck quantification and CD68 analysis, nonspecific binding was blocked with 2% bovine serum albumin (BSA, Sigma) and cells were permeabilized for 30 min with 0.1% Triton (Sigma) in PBS and afterwards incubated for 2h at with the primary antibody mouse anti-ASC (1:500, Biolegend)

or anti-human CD68 (clone 514H12; 1:100, Biorad), respectively. Subsequently, cells were washed and incubated for 1h with the appropriate fluorescence-conjugated secondary antibody: for ASC, Alexa Fluor® 647 anti-mouse IgG1 (clone RMG1-1; 1:200, Biolegend) was used and for CD68 Alexa Fluor® 488 anti-mouse IgD (1:100, Biolegend) then rinsed in PBS and incubated for 20 min with phalloidin-rhodamine (300 nM), rinsed again in PBS, and incubated for 10 min with DAPI (1µg/ml). All described procedures were performed at RT. Coverslips were mounted on slides with Fluoroshield without DAPI (Sigma). Images were acquired with a confocal microscopy (Leica TCS SP5 or TCS SP8) and analysed using Image J 2.0 software.

### *2.7. Western blot*

After cell priming, 25 µl of ice-cold cell lysis buffer supplemented with proteases inhibitor cocktail (Sigma) was added to cells in TCPS and 50 µl to cells in Ch scaffolds. Samples were incubated for 30 min on ice with frequent shacking, and then centrifuge for 15 min at 16000g at 4ºC. Supernatants were transferred to a fresh tube and were stored at -80ºC, until analysis. Proteins from the lysate were separated in 8-12% denaturing SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membrane (Bio-Rad). Membranes were probed with different antibodies: anti-NLRP3 mouse monoclonal (Cryo-2 clone; 1:1000; Adipogen), anti-IL-1β rabbit polyclonal antibody (H-153; 1:5000, Santa Cruz Biotechnology); anti-IL-1β (clone 2805; 1:5000, R&D); anti-β-actin mouse monoclonal antibody (C4; 1:10000, Santa Cruz Biotechnology) and mouse anti-human GAPDH (sc-47724; 1:5000, Santa Cruz Biotechnology). For BMDM experiments, western blot quantification was performed with Image Lab software (version 4.1 Copyright© BioRad laboratories) normalizing the values of each sample with the values of β-actin.

### *2.8. Degradation assay*

Chitosan scaffolds without cells were incubated at 37ºC / 120 rpm during 72h with complete medium to evaluate if the Ch extracts (or the Ch degradation products) were able to induce inflammasome activation. After 72h, chitosan extracts were collected and frozen at -80ºC until use. Chitosan scaffolds extracts were prepared at 0.1mg of scaffold to 1 ml of fluid, according to ISO 10993-5:2009 standard.

The effect of Ch extracts was assessed using human macrophages at day 7 of differentiation. For macrophage differentiation, cells were plated in TCPS at a confluence of 0.5 × 106 cells/well. Monocytes were cultured for 6 days in complete medium and 50ng/ml of M-CSF. After 6 days of differentiation the medium was changed, cells were washed and fresh medium without M-CSF was added. Cells were primed at day 7 for 4h at 37°C with 1 µg/ml of LPS or Ch extracts (Ch4% extracts or Ch15% extracts) working as the first step

signal of inflammasome activation, rinsed three times with Et-buffer and incubated in the same buffer at 37°C with 10 µM of nigericin (Sigma – Aldrich) or Ch extracts (Ch4% extracts or Ch15% extracts) during 20 min for NLRP3 activation, as the second step signal.

### *2.9. Statistical analysis*

Comparisons of multiple groups were analyzed by one-way analysis of variance ANOVA with Kruskal-Wallis test with Dunn's multiple comparisons test using Prism 6 software (GraphPad). P-values $<0.05$ were considered statistically significant.

## *3. RESULTS*

### *3.1. Effect of 3D Ch scaffolds with different degrees of acetylation reduce IL-1β release upon NLRP3 activation*

To assess the role of the inflammasome in the macrophage response to 3D Ch scaffolds, BMDMs from C57BL/6J mice were analyzed in response to 3D Ch scaffolds with different DAs (4% and 15%). BMDMs were plated in the Ch scaffolds at day 6 and primed at day 7 with LPS (1 μg/ml, 4h) being the subsequent NLRP3 activation achieved after incubation with nigericin (10 μM, 20 min). Interestingly, our results showed that 3D Ch scaffolds *per se* were not able to activate the inflammasome, and no IL-1β release was detected with Ch scaffolds without stimulus and even after LPS priming (Fig. 1A). In addition, the release of IL-1β after NLRP3 inflammasome activation with nigericin was significantly decreased in BMDMs plated in 3D Ch scaffolds with lower DA when compared with cells in TCPS (Fig. 1A). Cells cultured in 3D Ch scaffolds with 15% DA also showed a decrease in IL-1β release, although the difference was not statistically significant (Fig. 1A). To reject the idea that LPS or nigericin could be adsorbed by Ch scaffolds thus being less bioavailable to activate inflammasomes, cells were primed with LPS for 4h (previously incubated with Ch scaffolds) and the subsequent NLRP3 activation was achieved after incubation with nigericin (also previously incubated with Ch scaffolds) during 20 min. The results showed that prior incubation of LPS and nigericin with Ch scaffolds (Ch4% or Ch15%) did not affect their bioavailability to activate inflammasomes (data not shown).

As a control, we confirmed that the absence of ASC (*Pycard*$^{-/-}$) and Caspase-1 (*Caspase1/11*$^{-/-}$), impaired the release of IL-1β after NLRP3 activation in macrophages cultured in 3D Ch scaffolds (Fig. 1B).

We observed that when BMDMs were seeded in Ch scaffold, a considerable number of macrophages migrated and attached to the bottom of the well (Fig. 1C, right column). Therefore, to assess if the macrophages within the Ch scaffolds and the ones migrating to the well, will have a differential effect in NLRP3 activation, 24 hours after cell seeding the Ch scaffolds were changed to a new well and inflammasome priming and activation was performed on both sets of macrophages (the cells within the scaffolds and the cells that attached to the bottom of the wells). Similar results were obtained on both fractions (Fig. 1D), showing that Ch scaffolds were able to modulate macrophage response to NLRP3 inflammasome activation in both cells, the ones within and the ones outside the scaffold. The effect observed in the macrophages that migrate to the bottom of the well could be related with their short interaction with the scaffolds, but also due to the released mediators

by macrophages adherent to the scaffolds, or even due to degradation products released by the scaffolds.

Inflammasome activation leads to a type of lytic cell death known as pyroptosis [33]. Lactate dehydrogenase (LDH) is a cytosolic enzyme that is released into the culture medium following pyroptosis [53], therefore the presence of LDH in cell supernatants was determined. LDH leakage induced by inflammasome activation was lower in BMDMs seeded on 3D Ch scaffolds comparing with cells in TCPS, presenting the macrophages 39% less pyroptosis when cultured in Ch4% scaffolds than in TCPS (Fig. 1E). Finally, to reject the hypothesis that the decrease of IL-1β could be related with a low number of macrophages present in the Ch scaffolds, we decided to measure total intracellular LDH activity in LPS-primed macrophages cultured in TCPS and the Ch scaffolds. We found that the number of macrophages was higher in Ch scaffolds with lower DA compared with TCPS (Fig. 1F). We have measured the cell number after LPS priming in order to confirm that equal number of cells were in the scaffolds and TCPS before nigericin stimulation so that when inflammasome is trigger we have a similar number of cells in all the experimental conditions. It is important to clarify that LPS + nigericin is not a co-stimulation, but a serial stimulation. Thus, the LPS is the base for the priming time and then nigericin is added for a short time, which induces pyroptotic cell death. Variations in NLRP3 activation will lead to different degree of pyroptosis and different number of cells in the final cultures. This could be explained in base on previous studies demonstrating that Ch scaffolds lead to an increase in the metabolic activity of macrophages, comparing with cells in TCPS, proving that Ch scaffolds supports adhesion and development of macrophages [54]. This result confirms that the decrease of IL-1β is due to the modulatory effect of 3D Ch scaffolds with lower DA on macrophage function. Chitosan scaffolds with higher DA also induced a decrease of IL-1β, although with a lower effect when compared to Ch scaffolds with lower DA.

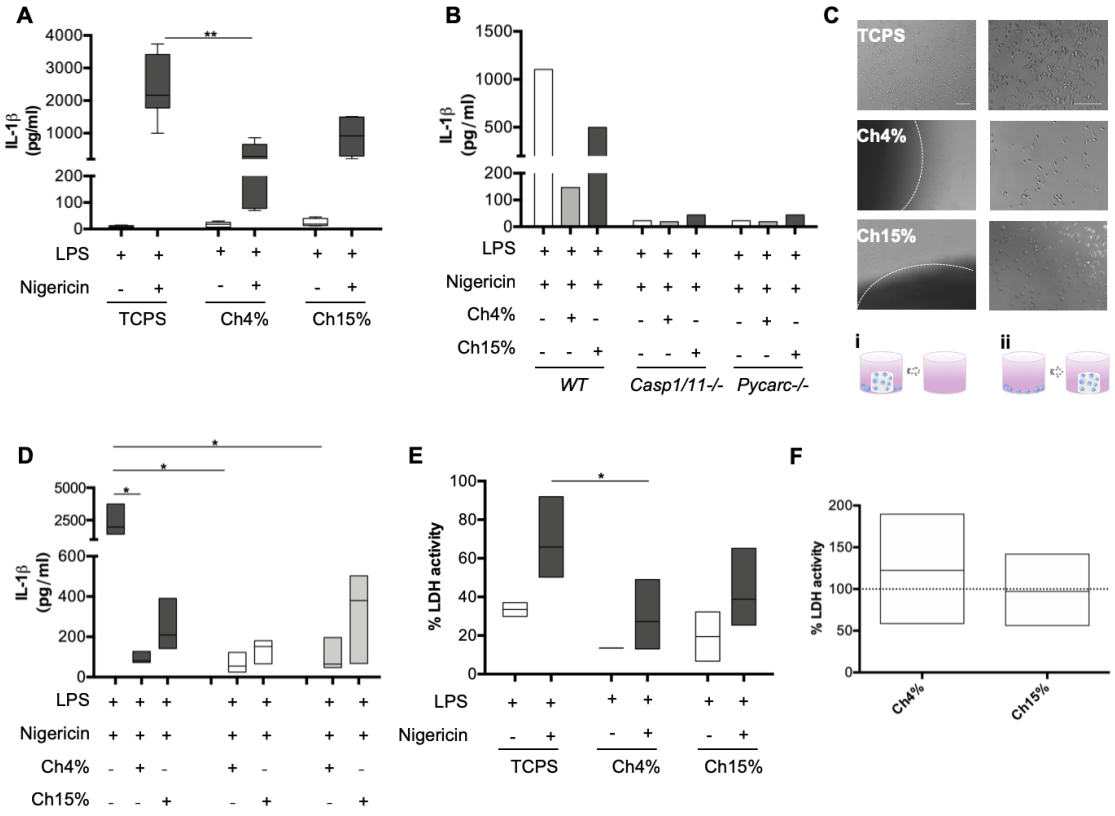



**Figure 1 | IL-1β release after NLRP3 activation is decreased in macrophages plated on 3D Ch scaffolds.** (A, B) ELISA for IL-1β release after NLRP3 activation in BMDMs from wild type mice (A, B), *Pycard*$^{-/-}$ or *Caspase1/11*$^{-/-}$ (B) cultured in tissue culture plates (TCPS) or 3D Ch scaffolds with different DAs (n>5 for A, n=1 for B). (C) Representative images of wild type BMDMs in culture. Scale bar=100μm; dotted line represents the edge of the Ch scaffold. (i,ii) Schematic representation of the experiment. i) Ch scaffolds were transferred to a new well 24h after cell seeding; ii) the inflammasome activation was performed as in A on both cell fractions. (D) ELISA for IL-1β release after transfer the scaffold to a new well (n=3). Chitosan scaffolds were transferred to a new well 24hrs after cell seeding and treated as in A. (White bars represents cells that have migrated from the respective Ch scaffolds to the bottom of the well and treated as in A); (Light Grey bars represents cells in the Ch scaffolds that were transferred to a new well and treated as in A). (E) Percentage of extracellular LDH activity measured in supernatants from wild type BMDMs treated as in A. (F) Percentage of LDH activity from cell lysates of from BMDMs cultured in different Ch scaffolds normalized to BMDMs cultured in TCPS (100%, indicated by a dashed line). Statistically significant differences: *p<0.05; **p<0.01 using Kruskal-Wallis test with Dunn's multiple comparisons test.

### *3.2. Chitosan scaffolds with lower degree of acetylation impair NLRP3 inflammasome oligomerization*

The effect of 3D Ch scaffolds on NLRP3 inflammasome oligomerization was analyzed through the staining of the ASC protein. Upon activation of NLRP3, ASC proteins oligomerize into fiber-like structures and the ASC filaments recruit caspase-1 leading to the formation of the inflammasome; staining of the large protein aggregates formed by ASC assembly makes possible the visualization of the inflammasome complex [55]. As shown in

the Fig. 2A, ASC oligomers (red dots) are mostly visualized near the nucleus. The number of ASC oligomers per cell was quantified with the Image J software. The images confirmed that macrophages cultured into 3D Ch scaffolds (4% and also 15%) were not able to induce inflammasome oligomerization, since no ASC oligomerization was detected after LPS priming (Fig. 2A, first column).

Since IL-1β release was strongly down-modulated in presence of 3D Ch scaffolds, we then confirmed that the formation of ASC oligomers followed the same trend. As depicted in Fig. 2B, BMDMs seeded in Ch scaffolds with 4% DA presented a significantly lower number of macrophages containing ASC oligomers, 20.35 ± 9.75 % when compared with cells in TCPS, 60.73 ± 17.44 % (Fig 2B). Macrophages seeded on 15% DA Ch scaffolds also presented a reduced number of cells with ASC oligomers, though the differences were not statistically significant compared to cells plated on TCPS (Fig 2B). After inflammasome activation, together with IL-1β release, oligomeric particles of inflammasomes are also released as particulate danger signals that could amplify the inflammatory response [56, 57]. We quantified extracellular ASC oligomers and the results showed a reduced number of ASC specks released after NLRP3 activation from macrophages plated in Ch scaffolds with lower DA comparing with TCPS and with Ch scaffolds with higher DA (Fig. 2C).

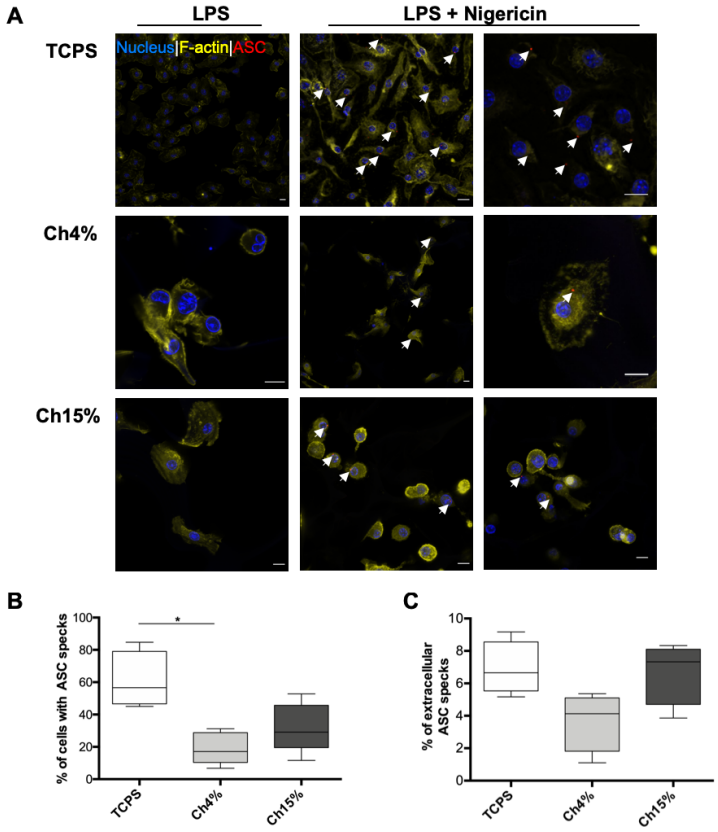



***Figure 2 | Chitosan scaffolds decrease the formation of ASC specks after NLRP3 activation.*** (A) Representative images of LPS-primed macrophages cultured in TCPS or Ch scaffolds with different DAs and

stimulated or not with nigericin as indicated, followed by staining of ASC (red), actin (yellow; phalloidin) and nuclei (blue; DNA-binding dye DAPI). Arrows indicate ASC oligomers. Scale bar=10μm. (B) Percentage of BMDMs with ASC specks quantified from images as in A, average data from n=4 independent experiments. (C) Percentage of extracellular ASC oligomers (n=4) from macrophages cultured and stimulated with LPS + Nigericin. Statistically significant differences: *p<0.05 using Kruskal-Wallis test with Dunn's multiple comparisons test.

### *3.3. Chitosan scaffolds affect NLRP3 inflammasome priming*

To assess the effect of 3D Ch scaffolds on inflammasome priming, we next studied pro-IL-1β and NLRP3 protein expression in BMDMs cultured on 3D Ch scaffolds and TCPS after LPS priming and NLRP3 activation by nigericin. For that, macrophages cultured in Ch scaffolds were lysed for Western blot analysis. As shown in Fig. 3A, scaffolds did not stimulate the expression of inflammasome components after LPS priming. In fact, we observed that the expression of pro-IL-1β after LPS priming was highly reduced in BMDMs seeded on both Ch scaffolds, being the differences statistically significant once again for Ch scaffolds with lower DA when compared with cells in TCPS (Fig. 3A, B). The results also showed that after nigericin stimulation most of pro-IL-1β from cells in TCPS and on Ch scaffolds with higher DA is lost in the cellular fraction, suggesting its release (Fig. 3A). On the other hand, in Ch scaffolds with lower DA we observed a slightly increase in the intracellular detection of pro-IL-1β after NLRP3 activation (Fig. 3A, B). In contrast, no differences were found in the transcriptional priming of NLRP3 when compared macrophages cultured in TCPS or Ch scaffolds (Fig. 3A, C). These results, together with the decrease of IL-1β release and the reduced number of ASC specking macrophages, suggest that 3D Ch scaffold with lower DA decrease the NLRP3 inflammasome activation.

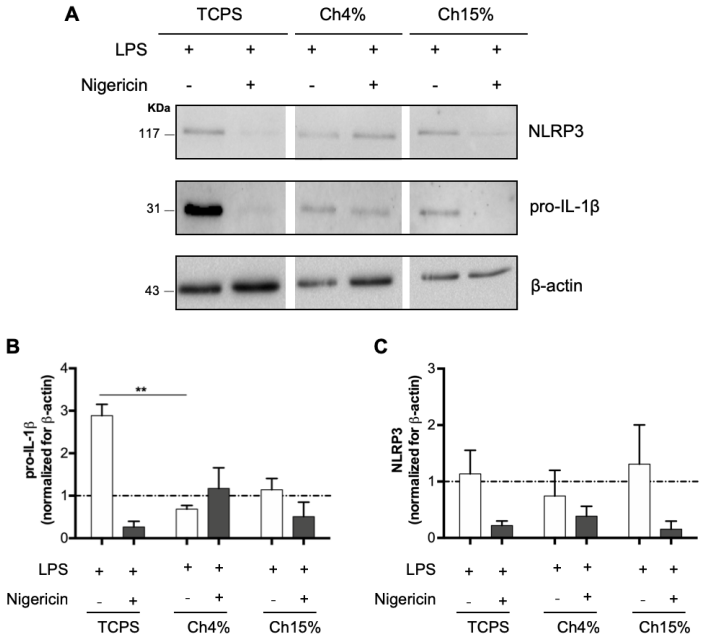



***Figure 3 | Pro-IL-1β, but not NLRP3, transcriptional priming is affected by Ch scaffolds.*** (A) Representative Western blot analysis of NLRP3 and pro-IL-1β in cell lysates of BMDMs primed for with LPS followed by no stimulation or stimulation of NLRP3 with nigericin; β-actin serves as a loading control throughout. (B, C) Quantification of pro-IL-1β (B) or NLRP3 (C) from n=3 independent blots as the one shown in panel A. Kruskal-Wallis with Dunn's multiple comparisons test. Statistically significant differences: **p<0.01.

### *3.4. NLRP3 inflammasome activation is impaired in human macrophages cultured on Ch scaffolds*

Human macrophages from "buffy coats" of healthy donors were cultured in 3D Ch scaffolds with different DA (4% and 15%). After 7 days of culture we confirmed that human macrophages cultured in Ch scaffolds were viable and expressed the monocyte/macrophage marker CD68 (Fig. 4A). As CD68 is a member of the scavenger receptor family with a role in phagocytosis, macrophages cultured in Ch scaffolds showed a CD68 distribution mostly in the adhesion region to the scaffolds, in contrast with cells in TCPS where the staining is distributed uniformly through the cell (Fig. 4A). We observed that human macrophages released a small amount of IL-1β during LPS priming when cultured in either TCPS or in Ch scaffolds (Fig. 4B). Since human monocytes exhibit an alternative one-step pathway of NLRP3 activation in response to LPS alone [58], and our macrophage cultures derive from monocytes, it could be feasible that some alternative NLRP3 activation could account for this release. However, after NLRP3 activation with nigericin human macrophages seeded in Ch scaffolds presented a decrease of IL-1β release, being statistically significant different for cells cultured in Ch scaffolds with higher DA (Fig. 4B).

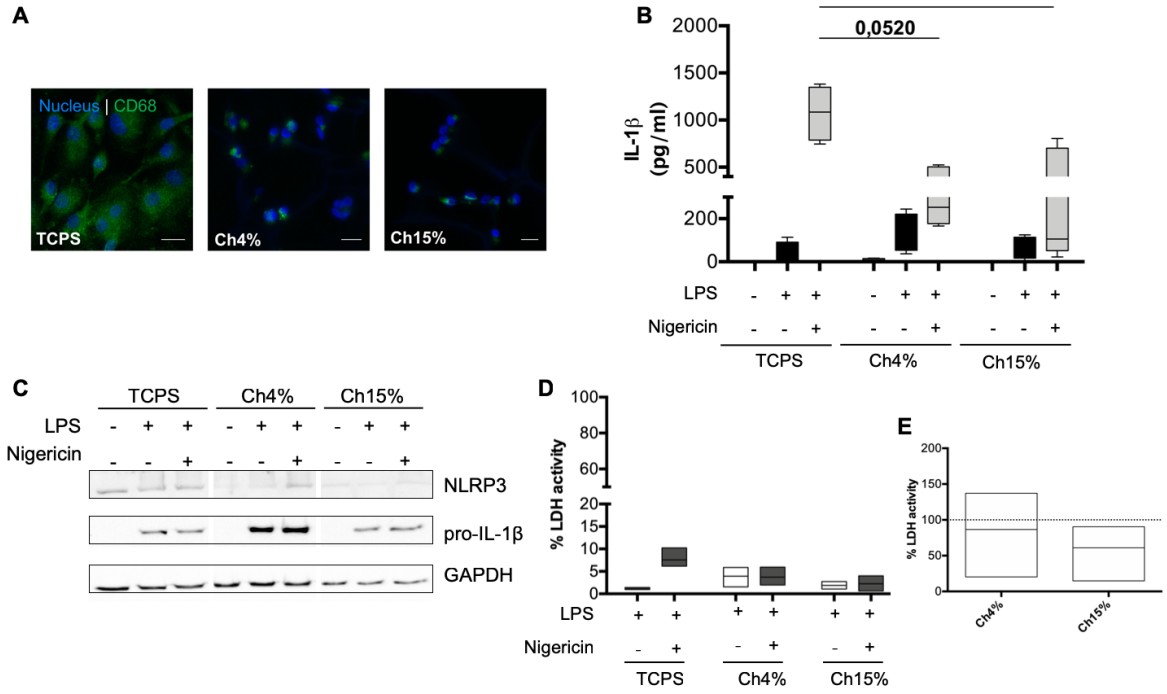



**Figure 4 | IL-1β release after NLRP3 activation in human macrophages cultured in Ch scaffolds.** (A) Representative image of CD68 expression on human macrophages after 7 days in culture, CD68 (green) and nuclei (blue; DNA-binding dye DAPI); scale bar = 20μm. (B) ELISA for IL-1β release after NLRP3 activation in human macrophages cultured in TCPS or Ch scaffolds with different degree of acetylation (n=5). (C) Representative blots of NLRP3 and pro-IL-1β in cell lysates of human macrophages primed with LPS followed by no stimulation or stimulation of NLRP3 with nigericin; GAPDH serves as a loading control throughout. (D) Percentage of LDH activity in the supernatant of human macrophages treated as in A and normalized to total intracellular LDH levels. (E) Percentage of LDH activity in lysates from Ch scaffolds, dotted line represents the LDH activity of macrophages cultured in TCPS. Statistically significant differences: *p<0.05 using Kruskal-Wallis test with Dunn's multiple comparisons test.

To assess if Ch scaffolds were able to influence the transcriptional priming step of NLRP3 inflammasome in human macrophages, NLRP3 and pro-IL-1β protein expression was analyzed in cell extracts. We observed a decrease of NLRP3 expression after LPS priming in both Ch scaffolds when compared to cells in TCPS (Fig. 4C). Surprisingly, human macrophages cultured in Ch scaffolds with lower DA showed an increase of pro-IL-1β after LPS priming, when compared with macrophages cultured in TCPS and Ch scaffolds with higher DA (Fig. 4C). The amount of pro-IL-1β protein did not change after nigericin stimulation (Fig. 4C), probably due to the low expression of NLRP3 that compromise the activation of NLRP3. These results confirmed the low amount of IL-1β released from macrophages cultured in Ch scaffolds. This is in line with the reduced pyroptosis found in human macrophages cultured in Ch scaffolds after NLRP3 activation (Fig. 4D). This result was not due to a lower number of macrophages present in the Ch scaffolds, as similar levels of LDH was found in macrophages lysates when cultured in TCPS and Ch scaffolds (Fig. 4E).

In order to assess if the Ch scaffold degradation products could influence macrophage behavior, the effect of Ch degradation products (extracts) on human macrophages was investigated and the results demonstrated that the extracts from both scaffolds (4% and 15%) were not able to induce a significant release of IL-1β when compared with the controls (Fig. 5 A, B).

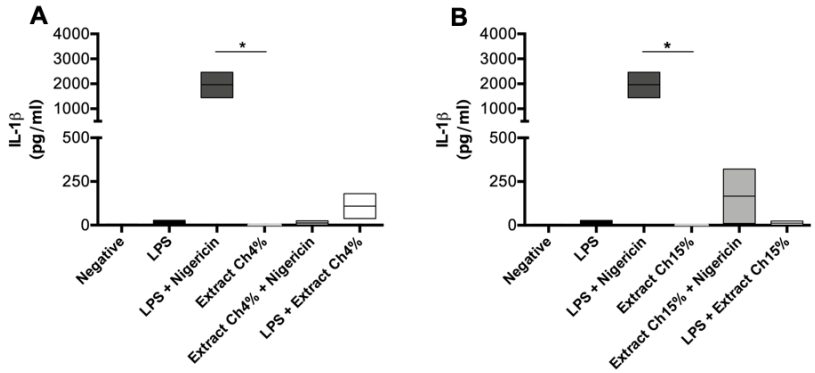



**Figure 5 | IL-1β release in human macrophages cultured in TCPS after NLRP3 inflammasome activation with chitosan degradation products.** (A) ELISA for IL-1β release after human macrophages priming with LPS or Ch4% extracts (first step signal) and subsequent NLPR3 activation with nigericin or Ch 4% extracts (second step signal) (n=3). (B) ELISA for IL-1β release after human macrophages priming with LPS or Ch15% extracts (first step signal) and subsequent NLPR3 activation with nigericin or Ch 15% extracts (second step signal) (n=3)). Statistically significant differences: *p<0.05 using Kruskal-Wallis test with Dunn's multiple comparisons test.

In addition, human macrophages cultured in Ch scaffolds with lower DA presented a decreased number of ASC specks (7.22 ± 2.74%) being this also observed for the macrophages cultured in Ch scaffolds with higher DA (6.64 ± 2.47%) when compared with cells plated in TCPS (29.27%±6.59), although this decrease was not statistically significant (Fig. 6 A, B). Taken together, our data show that 3D Ch scaffolds are able to down-regulate the activation of the NLRP3 inflammasome in human macrophages.

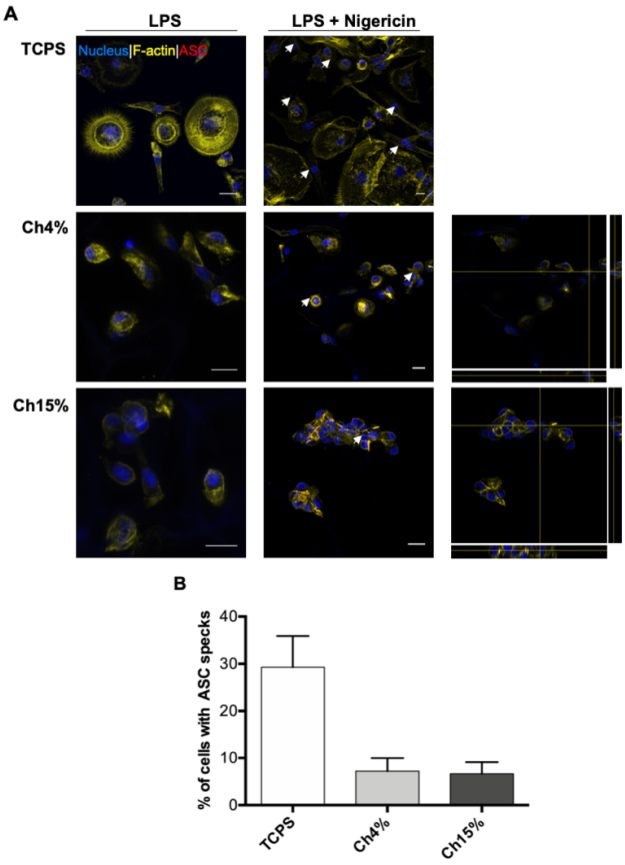



**Figure 6 | Chitosan scaffolds decrease the formation of ASC specks after NLRP3 activation in human macrophages.** (A) Representative images of LPS-primed human macrophages cultured in TCPS or Ch scaffolds with different DAs and stimulated or not with nigericin as indicated, followed by staining of ASC (red), actin (yellow; phalloidin) and nuclei (blue; DNA-binding dye DAPI). Arrows indicate ASC specks. Scale bar=20μm. Orthogonal views of cells within the Ch scaffold (right column). (B) Percentage of human macrophages with ASC specks quantified from images as in A, average data from n=3 independent experiments. Statistically significant differences: *$p<0.05$ using Kruskal-Wallis test with Dunn's multiple comparisons test.

## *4. DISCUSSION*

In this study, 3D Ch scaffolds with different DA were used to determine their modulation on NLRP3 inflammasome activation in the release of pro-inflammatory IL-1β from macrophage responding to macroscopic biomaterials. Our main finding is that Ch scaffolds decrease NLRP3 inflammasome priming and activation in *in vitro* cultured macrophages. This inhibition was found at the level of ASC oligomerization, IL-1β release and pyroptosis execution for both DAs studied.

Interestingly, although several studies have reported that Ch leads to inflammasome activation, by inducing $K^+$ efflux, reactive oxygen species production or lysosomal destabilization [24, 30] our data show that Ch 3D scaffolds were not able to activate the inflammasome in both mouse BMDMs and human macrophages. In addition, results also showed that the degradation products of Ch are not able to induce changes in the release of IL-1β in human macrophages. This could be due to the size of the material used in the different studies. It is known that implanted biomaterials with different sizes, induce distinctive immune responses [59]. Bueter et al. described an inverse association of the Ch particles size and inflammasome activity, suggesting that phagocytosis is required for inflammasome activation [24], and in our 3D scaffold-culture system, macrophages would not phagocyte Ch particles. Fong et al. found in U937 human macrophage-like cell line that biodegradable Ch microparticles selectively induced the release of IL-1β [60]. In this same cell line different Ch particles with controlled DA lead to either a type I IFN or inflammasome response, being this activation dependent on the extent of lysosomal rupture and cytosolic foreign body internalization [25]. Due to size disparity, macrophages are not able to phagocyte the 3D Ch scaffolds used in this study since they have a diameter of 11 mm and a thickness of 0.2 mm. Therefore, this work shows that the NLRP3 inflammasome is impaired when macrophages are cultured in Ch 3D macroscopic scaffolds. The impact of 3D macroscopic scaffolds on immune cells has not been well explored. A previous work showed differences in the cytokine secretion profile of from human macrophages interacting with different 3D structures. Chitosan scaffolds stimulated production of TNF-α and small amounts of IL-10 [61]. Different outcomes on macrophages behavior could be achieved changing the properties of the substrates. Our data show that material morphology and chemistry is an important aspect in biomaterial design that influence immune cell responses.

Interleukin-1β is a major pro-inflammatory cytokine with an important role in host-defense responses to infection and injury [62]. However, an exacerbated release of IL-1β can be related to severe inflammatory diseases [63]. Here, we found that the physical and chemical properties of Ch scaffolds were able to negatively modulate NLRP3 inflammasome activation in macrophages, decreasing the release of IL-1β and the degree of pyroptosis and suggesting a potential beneficial anti-inflammatory effect of implanted Ch scaffolds. Chitosan has been widely used to modulate the immune response, through the regulation of macrophage response [13, 20]. However, identifying the mechanisms behind these responses have been elusive. Our data show that 3D Ch scaffolds have a modulatory effect not only in cells inside in the scaffolds, but also in the cells that shared the same well, as a decrease of IL-1β was observed from macrophages that migrate or goes outside the scaffold when seeded. The decrease of IL-1β production was partially explained by a defect on the transcriptional priming, as murine, but not human, macrophages cultured in Ch scaffolds expressed less pro-IL-1β after LPS priming. This effect could be explained by the immunomodulatory capacity of Ch, previous described by us and others [13, 20, 52, 60, 64-66] demonstrating the production of several immune factors. Opposite results have been reported on the immune response obtained when Ch was present in different forms. Physical and chemical properties of Ch and the time of culture could dictate the type of the inflammatory response. We showed in a *in vivo* model of inflammation that 3D Ch scaffolds with higher DA increase the number of inflammatory cells recruited to the implant site the production of pro-inflammatory cytokines, such as IL-6 and TNF-α and also attracted mainly M1 macrophages, whereas 3D scaffolds with lower DA increase M2 macrophages in the implant site and IL-4 three days after of implantation [13, 47]. On the other hand, human macrophages cultured in Ch films with higher DAs presented a down-regulation of pro-inflammatory markers and cytokines, particularly TNF-α, and an increase of anti-inflammatory products such as IL-10 and TGF-β1 [20]. Oliveira M. *et al*. also showed that Ch films were not able to activate MAPK pathways ERK1/2 and JNK [66]. Tu J. *et al*. showed in LPS-primed Caco-2 cells that Ch nanoparticles were able to reduce inflammatory reaction decreasing cytoplasmic IκB-α degradation and nuclear NF-κB p65 levels [65]. However, it is noticeable that we found Ch scaffolds with lower degree of acetylation, to cause an increase pro-IL-1β in LPS-treated human macrophages. In any case, the release of IL-1β was still decreased in human macrophages after NLRP3 activation. This data confirms the importance of inflammasome activation for IL-1β release, even if enough pro-IL-1β is produced [67].

The NLRP3 inflammasome is also required for tissue repair and regeneration as reported in recent studies demonstrating its role in wound repair and liver regeneration [68-70]. However, when NLRP3 activation is exacerbated the correct resolution of inflammation does not occur and lead to fibrosis [71, 72], being high concentration of IL-1β negatively influence the healing process [73]. Our results are particularly important in this regard, as Ch scaffolds were able to decrease IL-1β release and pyroptotic cell death of macrophages. Consistently Ch scaffolds also decrease the number of ASC oligomers released, as these oligomers could be released as particulate danger signals [56, 57]. Therefore, the modulation of NLRP3 activity by Ch scaffolds can be in our opinion an interesting approach to improve biomaterial integration and enhance the outcomes of clinical conditions that involve tissue repair when excessive inflammation is present. Nevertheless, further studies in an *in vivo* model of tissue repair must be conducted to clearly assess the ability of the 3D Ch scaffolds to modulate the NLRP3 inflammasome activity leading to an improvement in biomaterial integration and subsequent tissue healing.

The overall inflammatory / anti-inflammatory effect of the scaffolds has been extensively investigated by us using a rodent air pouch model of inflammation where we have observed clear differences between both scaffolds: different DA (4% and 15%) hints distinctive macrophage polarization phenotype following material implantation. Chitosan scaffolds with higher DA induce a typical pro-inflammatory response mediated by M1 macrophages. On the other hand, Ch scaffolds with lower DA caused an inflammatory reaction characteristic of a macrophage M2 reparative response [13]. Following these results, we anticipated to observe clear differences in NLRP3 inflammasome activation in this study. These differences were not found, moreover the results on both DA were rather similar. We assume that these differences are related to the fact that in vivo, different cell types are present at the implant site and will secrete different mediators that will have an influence in the fate of macrophage polarization in response to the implanted material. In fact, there are clear differences between in vitro and in vivo models. The in vivo microenvironment is rather more complex, with a multiplicity of players that cannot be mimic in the in vitro studies.

Together our data have demonstrated that 3D Ch scaffolds impair NLRP3 inflammasome activity in macrophages resulting in a significant decrease of ASC oligomers formation, IL-1β release and pyroptotic cell death in both mouse and human macrophages. This effect was dependent on both, the transcriptional priming and activation of the NLRP3 inflammasome by macrophages cultured in Ch scaffolds and independently of the DA. Another important conclusion that can be taken from our results is that the inflammasome

activation is dependent of phagocytosis which does not occur with our 3D macroscopic scaffolds.

## *5. CONCLUSIONS*

We report for the first time that 3D Ch scaffolds modulate NLRP3 inflammasome activity. Interestingly, our results are in contrast with studies reported in the literature that indicate that Ch is a powerful activator of the NLRP3 inflammasome in nanoscale Ch products. Our studies that were performed in large scale Ch scaffolds, stressing out that the process of small Ch particles phagocytosis is pivotal in inflammasome assembly and activation. Our results revealed that macrophage culture in Ch scaffolds results in a significant decrease of IL-1β release, a decrease of ASC oligomer formation in both BMDMs and human macrophages after NLRP3 activation, and a decrease of LPS induced pro-IL-1β production in BMDMs and of NLRP3 in human macrophages. This effect was observed when macrophages were cultured in Ch scaffolds with different DAs. Interestingly the same behavior was observed for both DAs, which is in contrast with previous *in vivo* results of ours that demonstrated clear differences in the macrophage polarization profile, highlighting the importance of performing further studies using *in vivo* models. These findings provide an important understanding of the immunological properties of 3D Ch scaffolds, which will dictate a favorable fate for biomaterial integration.

## *ACKNOWLEDGMENTS*

This work was financed by: FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through FCT - Fundação para a Ciência e a Tecnologia in the framework of the project "Institute for Research and Innovation in Health Sciences" (POCI-01-0145-FEDER-007274) and NORTE-01-0145-FEDER-000012, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF), and supported by the European Research Council (ERC-2013-CoG 614578, to PP), the EU COST Program (BM1406, to PP) and the Ministerio de Economia, Industria y Competitividad–Fondo Europeo de Desarrollo Regional (project no. SAF2017-88276-R, to PP). Daniela P. Vasconcelos is funded by FCT PhD Programmes and by Programa Operacional Potencial Humano (POCH), BiotechHealth Programme (Doctoral Programme on Cellular and Molecular Biotechnology Applied to Health Sciences - reference PD/00016/2012), and through the PhD studentship PD/BD/114011/2015.

***REFERENCES***

[1] S.G. Santos, M. Lamghari, C.R. Almeida, M.I. Oliveira, N. Neves, A.C. Ribeiro, J.N. Barbosa, R. Barros, J. Maciel, M.C. Martins, R.M. Goncalves, M.A. Barbosa, Adsorbed fibrinogen leads to improved bone regeneration and correlates with differences in the systemic immune response, Acta biomaterialia 9(7) (2013) 7209-17. 10.1016/j.actbio.2013.04.008

[2] C. Intini, L. Elviri, J. Cabral, S. Mros, C. Bergonzi, A. Bianchera, L. Flammini, P. Govoni, E. Barocelli, R. Bettini, M. McConnell, 3D-printed chitosan-based scaffolds: An in vitro study of human skin cell growth and an in-vivo wound healing evaluation in experimental diabetes in rats, Carbohydr Polym 199 (2018) 593-602. 10.1016/j.carbpol.2018.07.057

[3] Y. Shu, Y. Yu, S. Zhang, J. Wang, Y. Xiao, C. Liu, The immunomodulatory role of sulfated chitosan in BMP-2-mediated bone regeneration, Biomater Sci 6(9) (2018) 2496-2507. 10.1039/c8bm00701b

[4] V. Vyas, T. Kaur, A. Thirugnanam, Chitosan composite three dimensional macrospheric scaffolds for bone tissue engineering, Int J Biol Macromol (2017). 10.1016/j.ijbiomac.2017.04.055

[5] V.P. Mantripragada, A.C. Jayasuriya, Bone regeneration using injectable BMP-7 loaded chitosan microparticles in rat femoral defect, Mater Sci Eng C Mater Biol Appl 63 (2016) 596-608. 10.1016/j.msec.2016.02.080

[6] D.P. Vasconcelos, M. Costa, N. Neves, J.H. Teixeira, D.M. Vasconcelos, S.G. Santos, A.P. Aguas, M.A. Barbosa, J.N. Barbosa, Chitosan porous 3D scaffolds embedded with resolvin D1 to improve in vivo bone healing, Journal of biomedical materials research. Part A 106(6) (2018) 1626-1633. 10.1002/jbm.a.36370

[7] S.A. Eming, T.A. Wynn, P. Martin, Inflammation and metabolism in tissue repair and regeneration, Science 356(6342) (2017) 1026-1030. 10.1126/science.aam7928

[8] P. Laurent, V. Jolivel, P. Manicki, L. Chiu, C. Contin-Bordes, M.E. Truchetet, T. Pradeu, Immune-Mediated Repair: A Matter of Plasticity, Frontiers in immunology 8 (2017) 454. 10.3389/fimmu.2017.00454

[9] S.A. Eming, T. Krieg, J.M. Davidson, Inflammation in wound repair: molecular and cellular mechanisms, The Journal of investigative dermatology 127(3) (2007) 514-25. 10.1038/sj.jid.5700701

[10] A. Sica, A. Mantovani, Macrophage plasticity and polarization: in vivo veritas, The Journal of clinical investigation 122(3) (2012) 787-95. 10.1172/JCI59643

[11] A. Vishwakarma, N.S. Bhise, M.B. Evangelista, J. Rouwkema, M.R. Dokmeci, A.M. Ghaemmaghami, N.E. Vrana, A. Khademhosseini, Engineering Immunomodulatory

Biomaterials To Tune the Inflammatory Response, Trends Biotechnol 34(6) (2016) 470-82. 10.1016/j.tibtech.2016.03.009

[12] Z. Julier, A.J. Park, P.S. Briquez, M.M. Martino, Promoting tissue regeneration by modulating the immune system, Acta biomaterialia 53 (2017) 13-28. 10.1016/j.actbio.2017.01.056

[13] D.P. Vasconcelos, A.C. Fonseca, M. Costa, I.F. Amaral, M.A. Barbosa, A.P. Aguas, J.N. Barbosa, Macrophage polarization following chitosan implantation, Biomaterials 34(38) (2013) 9952-9. 0.1016/j.actbio.2017.01.056

[14] D.P. Vasconcelos, M. Costa, I.F. Amaral, M.A. Barbosa, A.P. Aguas, J.N. Barbosa, Development of an immunomodulatory biomaterial: Using resolvin D1 to modulate inflammation, Biomaterials 53 (2015) 566-73. 10.1016/j.biomaterials.2015.02.120

[15] L. Zhang, Z. Cao, T. Bai, L. Carr, J.R. Ella-Menye, C. Irvin, B.D. Ratner, S. Jiang, Zwitterionic hydrogels implanted in mice resist the foreign-body reaction, Nature biotechnology 31(6) (2013) 553-6. 10.1038/nbt.2580

[16] T.D. Smith, R.R. Nagalla, E.Y. Chen, W.F. Liu, Harnessing macrophage plasticity for tissue regeneration, Adv Drug Deliv Rev 114 (2017) 193-205. 10.1016/j.addr.2017.04.012

[17] S. Franz, F. Allenstein, J. Kajahn, I. Forstreuter, V. Hintze, S. Moller, J.C. Simon, Artificial extracellular matrices composed of collagen I and high-sulfated hyaluronan promote phenotypic and functional modulation of human pro-inflammatory M1 macrophages, Acta biomaterialia 9(3) (2013) 5621-9. 10.1016/j.addr.2017.04.012

[18] H.M. Rostam, S. Singh, F. Salazar, P. Magennis, A. Hook, T. Singh, N.E. Vrana, M.R. Alexander, A.M. Ghaemmaghami, The impact of surface chemistry modification on macrophage polarisation, Immunobiology 221(11) (2016) 1237-46. 10.1016/j.imbio.2016.06.010

[19] J.R. Alhamdi, T. Peng, I.M. Al-Naggar, K.L. Hawley, K.L. Spiller, L.T. Kuhn, Controlled M1-to-M2 transition of aged macrophages by calcium phosphate coatings, Biomaterials (2018). 10.1016/j.biomaterials.2018.07.012

[20] M.I. Oliveira, S.G. Santos, M.J. Oliveira, A.L. Torres, M.A. Barbosa, Chitosan drives anti-inflammatory macrophage polarisation and pro-inflammatory dendritic cell stimulation, European cells & materials 24 (2012) 136-52; discussion 152-3. 10.22203/eCM

[21] C. Porporatto, I.D. Bianco, C.M. Riera, S.G. Correa, Chitosan induces different L-arginine metabolic pathways in resting and inflammatory macrophages, Biochemical and biophysical research communications 304(2) (2003) 266-72. 10.1016/S0006-291X(03)00579-5

[22] H.B.T. Moran, J.L. Turley, M. Andersson, E.C. Lavelle, Immunomodulatory properties of chitosan polymers, Biomaterials 184 (2018) 1-9. 10.1016/j.biomaterials.2018.08.054

[23] E.C. Carroll, L. Jin, A. Mori, N. Munoz-Wolf, E. Oleszycka, H.B.T. Moran, S. Mansouri, C.P. McEntee, E. Lambe, E.M. Agger, P. Andersen, C. Cunningham, P. Hertzog, K.A. Fitzgerald, A.G. Bowie, E.C. Lavelle, The Vaccine Adjuvant Chitosan Promotes Cellular Immunity via DNA Sensor cGAS-STING-Dependent Induction of Type I Interferons, Immunity 44(3) (2016) 597-608. 10.1016/j.immuni.2016.02.004

[24] C.L. Bueter, C.K. Lee, V.A. Rathinam, G.J. Healy, C.H. Taron, C.A. Specht, S.M. Levitz, Chitosan but not chitin activates the inflammasome by a mechanism dependent upon phagocytosis, The Journal of biological chemistry 286(41) (2011) 35447-55. 10.1074/jbc.M111.274936

[25] D. Fong, P. Gregoire-Gelinas, A.P. Cheng, T. Mezheritsky, M. Lavertu, S. Sato, C.D. Hoemann, Lysosomal rupture induced by structurally distinct chitosans either promotes a type 1 IFN response or activates the inflammasome in macrophages, Biomaterials 129 (2017) 127-138. 10.1016/j.biomaterials.2017.03.022

[26] J.M. Anderson, A. Rodriguez, D.T. Chang, Foreign body reaction to biomaterials, Seminars in immunology 20(2) (2008) 86-100. 10.1016/j.smim.2007.11.004

[27] S.N. Christo, K.R. Diener, J. Manavis, M.A. Grimbaldeston, A. Bachhuka, K. Vasilev, J.D. Hayball, Inflammasome components ASC and AIM2 modulate the acute phase of biomaterial implant-induced foreign body responses, Sci Rep 6 (2016) 20635. 10.1038/srep20635

[28] K. Schroder, J. Tschopp, The inflammasomes, Cell 140(6) (2010) 821-32. 10.1016/j.cell.2010.01.040

[29] F. Martinon, K. Burns, J. Tschopp, The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta, Molecular cell 10(2) (2002) 417-26. 10.1016/S1097-2765(02)00599-3

[30] C.L. Bueter, C.K. Lee, J.P. Wang, G.R. Ostroff, C.A. Specht, S.M. Levitz, Spectrum and mechanisms of inflammasome activation by chitosan, Journal of immunology 192(12) (2014) 5943-51. 10.4049/jimmunol.1301695

[31] A. Stutz, D.T. Golenbock, E. Latz, Inflammasomes: too big to miss, The Journal of clinical investigation 119(12) (2009) 3502-11. 10.1172/JCI40599

[32] F. Martinon, A. Mayor, J. Tschopp, The inflammasomes: guardians of the body, Annu Rev Immunol 27 (2009) 229-65. 10.1146/annurev.immunol.021908.132715

[33] S.K. Vanaja, V.A. Rathinam, K.A. Fitzgerald, Mechanisms of inflammasome activation: recent advances and novel insights, Trends Cell Biol 25(5) (2015) 308-15. 10.1016/j.tcb.2014.12.009

[34] O. Gross, C.J. Thomas, G. Guarda, J. Tschopp, The inflammasome: an integrated view, Immunol Rev 243(1) (2011) 136-51. 0.1111/j.1600-065X.2011.01046.x

[35] F.G. Bauernfeind, G. Horvath, A. Stutz, E.S. Alnemri, K. MacDonald, D. Speert, T. Fernandes-Alnemri, J. Wu, B.G. Monks, K.A. Fitzgerald, V. Hornung, E. Latz, Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression, Journal of immunology 183(2) (2009) 787-91. 10.4049/jimmunol.0901363

[36] G. Lopez-Castejon, N.M. Luheshi, V. Compan, S. High, R.C. Whitehead, S. Flitsch, A. Kirov, I. Prudovsky, E. Swanton, D. Brough, Deubiquitinases regulate the activity of caspase-1 and interleukin-1beta secretion via assembly of the inflammasome, The Journal of biological chemistry 288(4) (2013) 2721-33. 10.1074/jbc.M112.422238

[37] P. Palazon-Riquelme, J.D. Worboys, J. Green, A. Valera, F. Martin-Sanchez, C. Pellegrini, D. Brough, G. Lopez-Castejon, USP7 and USP47 deubiquitinases regulate NLRP3 inflammasome activation, EMBO Rep 19(10) (2018). 10.15252/embr.201744766

[38] L. Sborgi, S. Ruhl, E. Mulvihill, J. Pipercevic, R. Heilig, H. Stahlberg, C.J. Farady, D.J. Muller, P. Broz, S. Hiller, GSDMD membrane pore formation constitutes the mechanism of pyroptotic cell death, EMBO J 35(16) (2016) 1766-78. 10.15252/embj.201694696

[39] C.L. Evavold, J. Ruan, Y. Tan, S. Xia, H. Wu, J.C. Kagan, The Pore-Forming Protein Gasdermin D Regulates Interleukin-1 Secretion from Living Macrophages, Immunity 48(1) (2018) 35-44 e6. 10.1016/j.immuni.2017.11.013

[40] V. Petrilli, S. Papin, C. Dostert, A. Mayor, F. Martinon, J. Tschopp, Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration, Cell death and differentiation 14(9) (2007) 1583-9. 10.1038/sj.cdd.4402195

[41] I. Hafner-Bratkovic, P. Pelegrin, Ion homeostasis and ion channels in NLRP3 inflammasome activation and regulation, Curr Opin Immunol 52 (2018) 8-17. 10.1016/j.coi.2018.03.010

[42] H. Lima, Jr., L.S. Jacobson, M.F. Goldberg, K. Chandran, F. Diaz-Griffero, M.P. Lisanti, J. Brojatsch, Role of lysosome rupture in controlling Nlrp3 signaling and necrotic cell death, Cell Cycle 12(12) (2013) 1868-78. 10.4161/cc.24903

[43] S. Christo, A. Bachhuka, K.R. Diener, K. Vasilev, J.D. Hayball, The contribution of inflammasome components on macrophage response to surface nanotopography and chemistry, Sci Rep 6 (2016) 26207. 10.1038/srep26207

[44] A.L. Silva, C. Peres, J. Conniot, A.I. Matos, L. Moura, B. Carreira, V. Sainz, A. Scomparin, R. Satchi-Fainaro, V. Preat, H.F. Florindo, Nanoparticle impact on innate immune cell pattern-recognition receptors and inflammasomes activation, Seminars in immunology 34 (2017) 3-24. 10.1016/j.smim.2017.09.003

[45] A. Ho-Shui-Ling, J. Bolander, L.E. Rustom, A.W. Johnson, F.P. Luyten, C. Picart, Bone regeneration strategies: Engineered scaffolds, bioactive molecules and stem cells current

stage and future perspectives, Biomaterials 180 (2018) 143-162. 10.1016/j.biomaterials.2018.07.017

[46] L. Vachoud, N. Zydowicz, A. Domard, Formation and characterisation of a physical chitin gel, Carbohydrate Research 302(3-4) (1997) 169-177. 10.1016/S0008-6215(97)00126-2

[47] J.N. Barbosa, I.F. Amaral, A.P. Aguas, M.A. Barbosa, Evaluation of the effect of the degree of acetylation on the inflammatory response to 3D porous chitosan scaffolds, Journal of biomedical materials research. Part A 93(1) (2010) 20-8. 10.1002/jbm.a.32499

[48] S. Mariathasan, K. Newton, D.M. Monack, D. Vucic, D.M. French, W.P. Lee, M. Roose-Girma, S. Erickson, V.M. Dixit, Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf, Nature 430(6996) (2004) 213-8. 10.1038/nature02664

[49] K. Kuida, J.A. Lippke, G. Ku, M.W. Harding, D.J. Livingston, M.S. Su, R.A. Flavell, Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme, Science 267(5206) (1995) 2000-3. 10.1126/science.7535475

[50] F. Martin-Sanchez, C. Diamond, M. Zeitler, A.I. Gomez, A. Baroja-Mazo, J. Bagnall, D. Spiller, M. White, M.J. Daniels, A. Mortellaro, M. Penalver, P. Paszek, J.P. Steringer, W. Nickel, D. Brough, P. Pelegrin, Inflammasome-dependent IL-1beta release depends upon membrane permeabilisation, Cell death and differentiation 23(7) (2016) 1219-31. 10.1038/cdd.2015.176

[51] A.P. Cardoso, M.L. Pinto, A.T. Pinto, M.I. Oliveira, M.T. Pinto, R. Goncalves, J.B. Relvas, C. Figueiredo, R. Seruca, A. Mantovani, M. Mareel, M.A. Barbosa, M.J. Oliveira, Macrophages stimulate gastric and colorectal cancer invasion through EGFR Y(1086), c-Src, Erk1/2 and Akt phosphorylation and smallGTPase activity, Oncogene 33(16) (2014) 2123-33. 10.1038/onc.2013.154

[52] F. Castro, M.L. Pinto, A.M. Silva, C.L. Pereira, G.Q. Teixeira, M. Gomez-Lazaro, S.G. Santos, M.A. Barbosa, R.M. Goncalves, M.J. Oliveira, Pro-inflammatory chitosan/poly(gamma-glutamic acid) nanoparticles modulate human antigen-presenting cells phenotype and revert their pro-invasive capacity, Acta biomaterialia 63 (2017) 96-109. 10.1016/j.actbio.2017.09.016

[53] B.S. Cummings, R.G. Schnellmann, Measurement of cell death in mammalian cells, Curr Protoc Pharmacol Chapter 12 (2004) Unit 12 8. 10.1002/0471141755.ph1208s25.

[54] H.R. Caires, T. Esteves, P. Quelhas, M.A. Barbosa, M. Navarro, C.R. Almeida, Macrophage interactions with polylactic acid and chitosan scaffolds lead to improved recruitment of human mesenchymal stem/stromal cells: a comprehensive study with different immune cells, Journal of the Royal Society, Interface / the Royal Society 13(122) (2016). 10.1098/rsif.2016.0570

[55] F.I. Schmidt, A. Lu, J.W. Chen, J. Ruan, C. Tang, H. Wu, H.L. Ploegh, A single domain antibody fragment that recognizes the adaptor ASC defines the role of ASC domains in inflammasome assembly, J Exp Med 213(5) (2016) 771-90. 10.1084/jem.20151790

[56] A. Baroja-Mazo, F. Martin-Sanchez, A.I. Gomez, C.M. Martinez, J. Amores-Iniesta, V. Compan, M. Barbera-Cremades, J. Yague, E. Ruiz-Ortiz, J. Anton, S. Bujan, I. Couillin, D. Brough, J.I. Arostegui, P. Pelegrin, The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response, Nature immunology 15(8) (2014) 738-48. 10.1038/ni.2919

[57] B.S. Franklin, L. Bossaller, D. De Nardo, J.M. Ratter, A. Stutz, G. Engels, C. Brenker, M. Nordhoff, S.R. Mirandola, A. Al-Amoudi, M.S. Mangan, S. Zimmer, B.G. Monks, M. Fricke, R.E. Schmidt, T. Espevik, B. Jones, A.G. Jarnicki, P.M. Hansbro, P. Busto, A. Marshak-Rothstein, S. Hornemann, A. Aguzzi, W. Kastenmuller, E. Latz, The adaptor ASC has extracellular and 'prionoid' activities that propagate inflammation, Nature immunology 15(8) (2014) 727-37. 10.1038/ni.2913

[58] E. Vigano, C.E. Diamond, R. Spreafico, A. Balachander, R.M. Sobota, A. Mortellaro, Human caspase-4 and caspase-5 regulate the one-step non-canonical inflammasome activation in monocytes, Nat Commun 6 (2015) 8761. 10.1038/ncomms9761

[59] C.A. Da Silva, C. Chalouni, A. Williams, D. Hartl, C.G. Lee, J.A. Elias, Chitin is a size-dependent regulator of macrophage TNF and IL-10 production, Journal of immunology 182(6) (2009) 3573-82. 10.4049/jimmunol.0802113

[60] D. Fong, M.B. Ariganello, J. Girard-Lauziere, C.D. Hoemann, Biodegradable chitosan microparticles induce delayed STAT-1 activation and lead to distinct cytokine responses in differentially polarized human macrophages in vitro, Acta biomaterialia 12 (2015) 183-194. 10.1016/j.actbio.2014.10.026

[61] C.R. Almeida, T. Serra, M.I. Oliveira, J.A. Planell, M.A. Barbosa, M. Navarro, Impact of 3-D printed PLA- and chitosan-based scaffolds on human monocyte/macrophage responses: unraveling the effect of 3-D structures on inflammation, Acta biomaterialia 10(2) (2014) 613-22. 10.1016/j.actbio.2013.10.035

[62] C.A. Dinarello, Biologic basis for interleukin-1 in disease, Blood 87(6) (1996) 2095-147.

[63] C.A. Dinarello, Interleukin-1 in the pathogenesis and treatment of inflammatory diseases, Blood 117(14) (2011) 3720-32. 10.1182/blood-2010-07-273417

[64] C.D. Hoemann, G. Chen, C. Marchand, N. Tran-Khanh, M. Thibault, A. Chevrier, J. Sun, M.S. Shive, M.J. Fernandes, P.E. Poubelle, M. Centola, H. El-Gabalawy, Scaffold-guided subchondral bone repair: implication of neutrophils and alternatively activated arginase-1+ macrophages, Am J Sports Med 38(9) (2010) 1845-56. 10.1177/0363546510369547

[65] J. Tu, Y. Xu, J. Xu, Y. Ling, Y. Cai, Chitosan nanoparticles reduce LPS-induced inflammatory reaction via inhibition of NF-kappaB pathway in Caco-2 cells, Int J Biol Macromol 86 (2016) 848-56. 10.1016/j.ijbiomac.2016.02.015

[66] M.I. Oliveira, M.L. Pinto, R.M. Goncalves, M.C.L. Martins, S.G. Santos, M.A. Barbosa, Adsorbed Fibrinogen stimulates TLR-4 on monocytes and induces BMP-2 expression, Acta biomaterialia 49 (2017) 296-305. 10.1016/j.actbio.2016.11.034

[67] M.M. Gaidt, V. Hornung, Alternative inflammasome activation enables IL-1beta release from living cells, Curr Opin Immunol 44 (2017) 7-13. 10.1016/j.coi.2016.10.007

[68] E.M. Weinheimer-Haus, R.E. Mirza, T.J. Koh, Nod-like receptor protein-3 inflammasome plays an important role during early stages of wound healing, PloS one 10(3) (2015) e0119106. 10.1371/journal.pone.0119106

[69] H. Ito, A. Kanbe, H. Sakai, M. Seishima, Activation of NLRP3 signalling accelerates skin wound healing, Exp Dermatol 27(1) (2018) 80-86. 10.1111/exd.13441

[70] T. Ando, H. Ito, A. Kanbe, A. Hara, M. Seishima, Deficiency of NALP3 Signaling Impairs Liver Regeneration After Partial Hepatectomy, Inflammation 40(5) (2017) 1717-1725. 10.1007/s10753-017-0613-6

[71] X. Ouyang, A. Ghani, W.Z. Mehal, Inflammasome biology in fibrogenesis, Biochimica et biophysica acta 1832(7) (2013) 979-88. 10.1016/j.bbadis.2013.03.020

[72] C.M. Artlett, Inflammasomes in wound healing and fibrosis, The Journal of pathology 229(2) (2013) 157-67. 10.1002/path.4116

[73] X. Wang, T. Friis, V. Glatt, R. Crawford, Y. Xiao, Structural properties of fracture haematoma: current status and future clinical implications, J Tissue Eng Regen Med 11(10) (2017) 2864-2875. 10.1002/term.2190

# CHAPTER IV

## ORIGINAL RESEARCH WORK

### DEVELOPMENT OF A STRATEGY THAT WILL LEAD TO A FASTER RESOLUTION OF THE IMMUNE RESPONSE TO BIOMATERIALS

**ARTICLE II**

"Development of an immunomodulatory biomaterial: Using resolvin D1 to modulate inflammation." (2015). Biomaterials, 53.

**Development of an immunomodulatory biomaterial: Using resolvin D1 to modulate inflammation**

Daniela P. Vasconcelos[a,b,c], Madalena Costa[a,d], Isabel F. Amaral [b,c], Mário A. Barbosa[a,b,c], Artur P. Águas[a,d], Judite N. Barbosa[a,b,c,*]

[a]ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Portugal.

[b]Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal.

[c]INEB, Instituto de Engenharia Biomédica, Universidade do Porto, Porto, Portugal.

[d]UMIB–Unit for Multidisciplinary Biomedical Research of ICBAS, Universidade do Porto, Porto, Portugal.

**ABSTRACT**

In our search for immunomodulatory biomaterials capable of modulating the inflammatory response through M2 macrophage polarization, we report here on a new strategy that resulted from the incorporation of resolvin D1 (RvD1), a pro-resolution lipid mediator in porous 3D chitosan (Ch) scaffolds, followed by its lyophilization. We have investigated the inflammatory response caused by this biomaterial *in vivo* using a mouse air-pouch model of inflammation. We found that this developed material caused a decrease in inflammatory cells recruited to the implant site, together with higher numbers of F4/80+/CD206+ cells (M2 macrophages) and lower numbers of F4/80+/CCR7+ cells (M1 macrophages). It also induced a general decrease in pro-inflammatory cytokines and caused a decrease in the inflammatory cells observed around and within the implanted scaffolds, when compared with Ch alone or Ch not submitted to lyophilization after RvD1 incorporation. Our results demonstrate that we were able to develop an immunomodulating biomaterial that triggers a shift in the macrophage response towards a M2 reparative response that will be advantageous for the host.

***KEYWORDS***

Macrophages; immune response; chitosan; immunomodulation; resolvin

## 1. INTRODUCTION

Biomaterial scaffolds are central to many tissue engineering and regenerative medicine strategies as they create a space for tissue growth and provide support for cell adhesion and migration. However, biomaterial implantation results in unavoidable injury and an inflammatory response, which can impair integration with the host and tissue repair/regeneration [1]. The persistence of an inflammatory stimulus results in chronic inflammation that is a hallmark of the non-healing wound. Therefore, successful tissue repair and regeneration requires the coordinate expression of both inflammation and resolution of inflammation [2]. The magnitude and duration of acute inflammation are controlled by active resolution programs involving specialized pro-resolving lipid mediators that have potent anti-inflammatory and pro-resolving actions and thus actively drive termination of inflammation [3,4].

Lipid mediators of resolution of inflammation, including resolvins, protectins, lipoxins and maresins, are biosynthesized during the resolution phase of acute inflammation. These mediators exert their anti-inflammatory actions by inhibiting formation of free oxygen radicals by leukocytes, decreasing transendothelial migration of these cells, stimulating nonphlogistic recruitment of monocytes and by enhancing macrophage clearance of apoptotic leukocytes [4,5]. Based on previous studies of ours using different pro-resolution mediators we have decided to use Resolvin D1 (RvD1) [6]. Resolvin D1 is derived from u-3-PUFAs docosahexaenoic acid (DHA). In the presence of aspirin, ω-3-PUFAs are converted to the aspirin triggered form of resolvin through a cyclooxygenase-2 dependent pathway [7]. Resolvins induce the hallmark functions of resolution of inflammation, including prevention of neutrophil penetration, phagocytosis of apoptotic neutrophils and enhanced clearance of inflammation within the lesion and thus promoting tissue regeneration [4].

Macrophages have a key role in the outcome of the healing process following biomaterial implantation [8]. Macrophage lineage displays high plasticity and pivotal roles in inflammation [3]. In response to external cues, these cells change their activation state. Inflammatory cytokines and chemokines at the inflamed biomaterial implantation site typically drive the differentiation of M1 macrophages in the early biomaterial healing response. Dampening of these inflammatory signals is often disturbed due to the presence of the biomaterial resulting in a persistent inflammatory environment and leading to the well-known foreign body response. Persistent macrophage polarization into M1 is often associated with fibrotic and scar tissue formation in the biomaterial integration process. Thus, to enable a favorable healing response associated with functional tissue formation,

putative activation of inflammatory M1 macrophages and favor differentiation towards M2 wound healing/regulatory macrophages differentiation [8-12].

Previous results of ours revealed that successive local administrations of RvD1 enables the modulation of the inflammatory response to Ch scaffolds through M2 macrophage polarization [6]. The purpose of this study was to develop an immunomodulatory biomaterial through the incorporation of RvD1 into Ch 3D porous scaffolds in order to obtain a continuous release of this mediator locally. The data of the current investigation indicate that this strategy provides *in vivo* control of macrophage phenotypes which may be of great value in regenerative medicine applications. Our main goal is to develop an immunomodulatory biomaterial capable of promoting tissue regeneration, exploring the link between inflammation resolution and tissue regeneration. Important clinical endpoints are enhanced functional tissue regeneration and improved device integration and biocompatibility within the host.

## 2. MATERIALS & METHODS

### *2.1. Chitosan purification and characterization*

Squid pen Ch (ref. 114, Batch No. S4; DA ~2%) was supplied by Mahtani Chitosan Pvt. Ltd. and subsequently purified by filtration of Ch acidic solution and subsequent alkali precipitation. Chitosan with a degree of acetylation (DA) of 15% was prepared by N-acetylation of the former, according to Vachoud *et al*. [13], in a water/acetic acid/1,2-propanediol solution, using acetic anhydride as reactive. Following acetylation, a DA of 15.71 ± 0.52, weight-average molecular weight (Mw) of 8.3 ± 0.9 x $10^5$ and polydispersity index (PDI) of 1.3 ± 0.1, was found, as previously described [14]. Ch with DA 15% revealed endotoxin levels below 0.1EU/mL, respecting the FDA guidance documents for implantable devices, as determined using the Limulus Amebocyte Lysate test (QCL-1000® test, Cambrex) in Ch water extracts [15].

### *2.2. Preparation of Ch 3D scaffolds and incorporation of resolvin D1 (RvD1)*

The 3D porous scaffolds were prepared from degassed 2% w/v Ch solutions in 0.2 M acetic acid via thermally induced phase separation (-20ºC; 24 h) and subsequent sublimation of the ice crystals. Following lyophilization (Labconco 7420031; -80ºC; 0.2 mbar; 72 h) the resultant scaffolds were cut in discs with 8.5 mm in diameter and 2 mm in thickness. The scaffolds were then submitted to sterilization in an ethanol series: immersed in absolute ethanol (B Braun) for 1 h and then sequentially in 70% (v/v) for 30 min, 50% (v/v) and 25% (v/v) ethanol for 10 min each. Afterwards, Ch scaffolds were washed twice with ultrapure water (Milli Q) for 10 min. Scaffolds were then frozen (-20ºC; 24 h) and lyophilized for the second time (-80ºC; 0.2 mbar; 48 h), under sterile conditions (scaffolds were placed in 24 well plates at the freeze dryer with a filter membrane of 0.22 μm).

The incorporation of RvD1 in the Ch scaffolds was performed after their preparation and sterilization by an embedding technique under sterile conditions in a flow hood chamber. An ethanolic solution of RvD1 from Cayman Chemical (molecular weight 376,4865; pKa 1/4 4.58; water solubility: approximately 0.05 mg/mL at 25ºC or PBS pH 7,4) with a concentration of 1.66 ng/ml was prepared according to the provider instructions. A volume of 60 ml of the RvD1 solution was added drop-wise with a micropipette into the scaffold which was then lyophilized again (-80ºC; 0.2 mbar; 24 h). A control group was also prepared by adding 60 ml of the RvD1 solution using the same technique, but the scaffolds were not submitted to lyophilization after incorporation of RvD1. In this control group the scaffolds were lyophilized again prior to RvD1 incorporation (-80ºC; 0.2 mbar; 24 h), so that scaffolds of the two experimental groups are subjected to the same number of lyophilizations.

### *2.3. Characterization of 3D scaffolds by scanning electron microscopy (SEM)*

The morphology of the scaffolds was investigated by SEM in transversal and longitudinal cross-sections. The materials were analyzed after preparation and also after incorporation of RvD1 and subsequent lyophilization. The samples were coated with Au/Pd thin film, by sputtering, using the SPI Module Sputter Coater equipment and observed using a High resolution (Schottky) Environmental Scanning Electron Microscope with X-Ray Microanalysis and Electron Backscattered Diffraction analysis: Quanta 400 FEG ESEM/EDAX Genesis X4M.

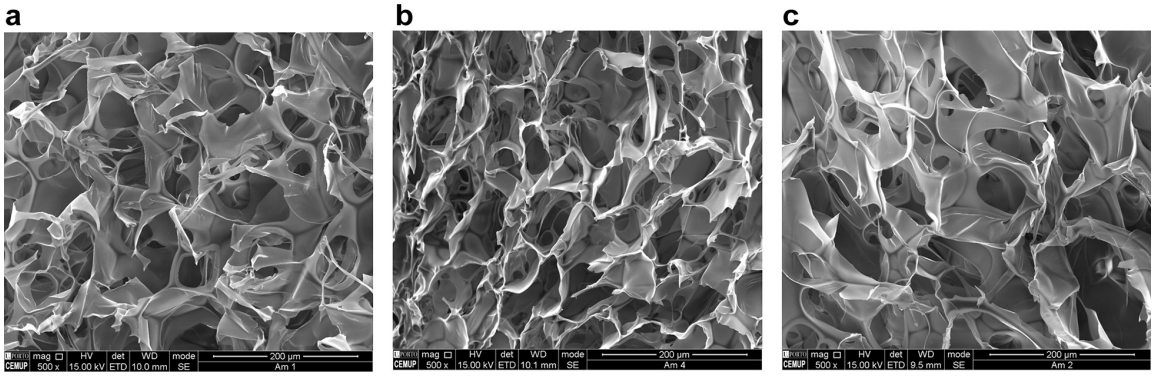



**Figure 1 | SEM micrographs of 3D porous scaffolds.** SEM micrographs of 3D porous scaffolds. (a) Ch 15%; (b) Ch 15% + RvD1; (c) Ch 15% + RvD1 Lyophilized. Scale bar: 200 μm.

### *2.4. Release studies of resolvin D1*

The release kinetics of RvD1 was investigated both for the lyophilized and non- lyophilized scaffolds after incorporation of RvD1, by immersing them in 3 mL of Dulbecco's phosphate-buffered saline (DPBS) at 37ºC under mild agitation (40 rpm). Samples were collected at the following time points: 0.5; 1; 2; 4; 6; 10; 24; 48 and 72 h. At each time point 1.5 mL of the solution was collected and replaced by the same volume of fresh DPBS. Quantitative analyses of RvD1 released was performed using a commercially available kit for the detection of RvD1 (Resolvin D1 Enzyme Immunoassay, Cayman Chemical). The results are presented as cumulative percentage of RvD1 mass released.

### *2.5. Animal model of inflammation*

The air-pouch model of inflammation was used according to the method of Sedwick *et al*. [16] as adapted by Castro *et al*. [17]. Briefly, mice were anaesthetized by intramuscular injection of ketamine (Ketalar, Parke-Davis Co., Spain; 4.0-8.0 mg/ kg of weight) and xylazine (Rompum, Bayer Co., Portugal; 0.8-1.6 mg/kg) and subsequently injected subcutaneously in the dorsal area with 5 mL of sterile air to induce the formation of an air pouch. A second subcutaneous injection of 3 mL of sterile air was performed 5 days later

to reinforce the air pouch, and 24 h after the scaffolds were implanted. Mice were anaesthetized as described above and the skin covering the air pouch area was shaved and cleaned. A surgical incision was made, the Ch DA 15% scaffolds were placed inside the air pouch and the incision was sutured. A single scaffold was implanted in each animal. For each experimental group, 6 male BALB/c mice (Charles River, Spain) with 7 weeks of age were used. Three experimental groups were used: One group with Ch DA 15% only (Ch 15%); one group with Ch DA 15% and RvD1 100 ng/mL lyophilized (Ch 15% + RvD1 Lyophilized); and one group with Ch DA 15% and RvD1 100 ng/mL not submitted to lyophilization (Ch 15% + RvD1).

The procedures involved in the animal model were approved by the in-house ethics committee and also by the Portuguese official authority responsible for animal welfare and experimentation (Direção Geral de Veterinária, DGV).

### *2.6. Inflammatory exudates*

The exudates were recovered from the mouse air-pouches 4 days after implantation. The mice were euthanized with a lethal injection of ketamine (Ketalar, Parke-Davis Co., Spain) and xylazine (Rompum, Bayer Co). Harvesting of inflammatory exudates was done by washing the air pouch cavity with 4 mL of PBS. The suture area was clumped, and 4 mL of PBS were injected using a 26-gauge needle (B Braun) in the air pouch at the caudal region. The pouches were then gently agitated at the sides to facilitate mixing. The recovery of the lavage fluid was done using a 20-gauge needle (B Braun) with the needle opening turned to the inner of the pouch. On average 2 mL of the lavage fluid were collected back in each aspiration. When the exudates were collected the pouches still contained the scaffolds.

### *2.7. Explants*

Explantion occurred immediately after the recovery of the inflammatory exudates (4 days after implantation). The sutures were cut, and the wound edges separated; the scaffolds were then carefully removed from the pouches and fixed in 4% formaldehyde (Panreac) for histological analysis.

### *2.8. Flow cytometry*

The following antibodies, all from R&D Systems, were used in this study: phycoerythrin (PE)-labelled anti-mouse F4/80 (clone 521204, 4:50 μl), allophycocyanin-labelled anti-mouse CCR7 (clone 4B12 7:50 μl), Alexa Fluor®488 labelled anti-mouse MMR/CD206 (3:50 μl). The isotype controls PE-labelled IgG2A (clone 54447), APC-labelled IgG2A (54447) and Alexa Fluor®488-labelled IgG, were used as negative controls to define background staining at corresponding concentrations.

The exudates were filtered through 40 mm nylon mesh (BD Biosciences) to remove cell clumps and spun at 1200 rpm for 5 min at 4ºC. Supernatants were removed, and cell pellets re-suspended in 1xPBS/0.5%BSA/0.1%Azide. Single-cell suspensions were preincubated with Fc-receptor-blocking antibodies (Miltenyi Biotec) for 10 min at 4ºC. Labelling was performed in a final volume of 50 ml with the indicated fluorescently conjugated antibodies for 30 min at 4ºC in the dark. Cells were then washed three times with staining buffer and transferred to FACS tubes for analysis by flow cytometry. Fluorescence was measured using FACS Calibur flow cytometer (BD biosciense) with Cell Quest software, and 10,000 events were

### *2.9. Cytokine production*

A commercially available cytokine array of 40 cytokine proteins (Mouse Cytokine Array Panel A; R&D Systems; Catalogue #ARY006) was used to evaluate the relative levels of cytokine production in the inflammatory exudates. A pool of 6 inflammatory exudates of each experimental group was prepared for this determination, and 1 mL of the prepared pooled was used. Data shown are from 5 min exposure in Chemidoc XRS+ (BioRad). Quantification of the results was generated by quantifying the mean spot pixel density from the array using image software analyses (ImageLab 4.1; BioRad). Briefly, the pixel intensities gathered from the array spots are obtained using the volume tools option of the software. We have defined an area of interest of the reference spots by surrounding it with a circle, and then equal circles were used for all spots of the array. Afterwards, the circles were analysed, and the densities of signals were normalized with the background.

### *2.10. Histological analysis*

The explanted scaffolds were processed and embedded in paraffin wax, and cut in thin sections of 3 mm. Three sections of each scaffold were prepared and stained using standard Masson trichrome. The stained sections were visualized using light microscopy (Zeiss, Axiovert 200M) under 10x magnification.

### *2.11. Statistical analysis*

Statistical analysis was performed using the non-parametric test Mann-Whitney with Graph Pad v6.02. for Windows. A value of $p<0.05$ was considered statistically significant: *$p<0.05$; **$p<0.01$; ***$p<0.001$. Data are presented showing individual groups and respective mean ± SEM.

## *3. RESULTS*

### *3.1. Characterization of Ch 3D scaffolds*

In accordance to previous results Ch scaffolds revealed a highly porous and homogeneous microstructure with interconnected pores with diameters in the range of 100 μm [14]. This was observed both for the Ch scaffolds immediately after its preparation and for those subjected to lyophilization after RvD1 incorporation. The results obtained by SEM analyses revealed that lyophilization after RvD1 incorporation did not affect the microstructure (Fig. 1).

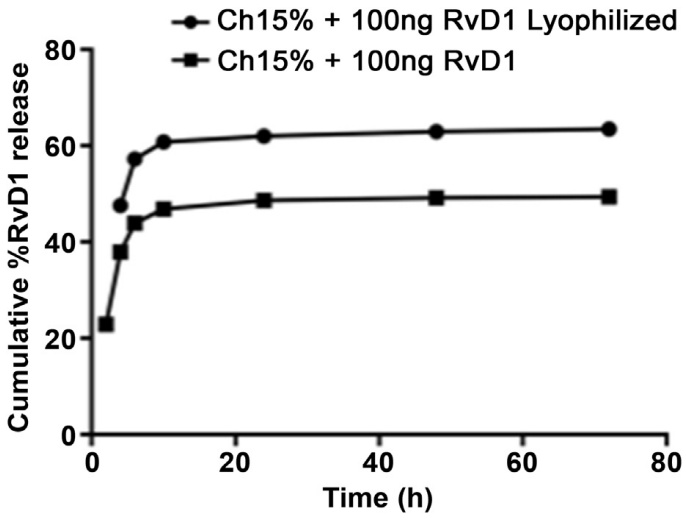



**Figure 2 | Release studies of Resolvin D1 (RvD1) from the Ch scaffolds.** The release of RvD1 was investigated both for scaffolds lyophilized and non-lyophilized after its incorporation. The results are presented as cumulative percentage of RvD1 mass released.

### *3.2. Release of resolvin D1 from Ch scaffolds*

RvD1 was embedded on the 3D porous Ch scaffolds by adding it drop-wise with a micropipette, followed by lyophilization. We have also characterized Ch scaffolds that were not submitted to lyophilization after the incorporation of RvD1. The release profile of RvD1 was then assessed in both groups of samples, and the results are presented in Fig. 2. Release results showed for both cases an initial burst period that lasted for 10 h, after which a plateau was reached, with RvD1 being released in reduced amounts until the end of the study (72 h). Nevertheless, we observed some differences in the two groups of samples. The group with lyophilized RvD1 presented a delay in the release of RvD1 but afterwards released higher concentrations of RvD1 when compared with the non-lyophilized samples.

### *3.3. Flow cytometry*

The inflammatory exudates were collected from the mouse air-pouches 4 days after the implantation of the scaffolds and analyzed by flow cytometry. Macrophages were detected using F4/80, a commonly used murine macrophage marker; the M1 and M2 subpopulations of macrophages were identified by CCR7 and CD206 markers respectively. Dot plots from a representative experiment are depicted in Fig. 3. The results presented in Fig. 4a show a decrease in the total number of inflammatory cells recruited to the implant site when RvD1 was incorporated in the Ch scaffolds, being this decrease more noticeable for scaffolds subjected to lyophilization after RvD1 incorporation. The use of phenotypic markers of the M1 and M2 macrophage subpopulations showed that the inflammatory exudates collected from air pouches with scaffolds embedded with RvD1 and followed by lyophilization presented higher percentage of CD206+ cells (M2 macrophages), together with a lower percentage of CCR7+ cells (M1 macrophages) in comparison with the other experimental groups, being these differences always statistically significant (Fig. 4c and d). These results can be confirmed in Fig. 4e that displays the ratio of M2/M1 positive cells and shows a marked increase in the ratio of M2/M1 cells for scaffolds embedded with RvD1 and followed by lyophilization when compared with the other experimental groups. These data are indicative of an M2 macrophage phenotypic response, which is in contrast with the response caused by Ch alone or Ch with RvD1 without lyophilization which triggered an M1 macrophage response.

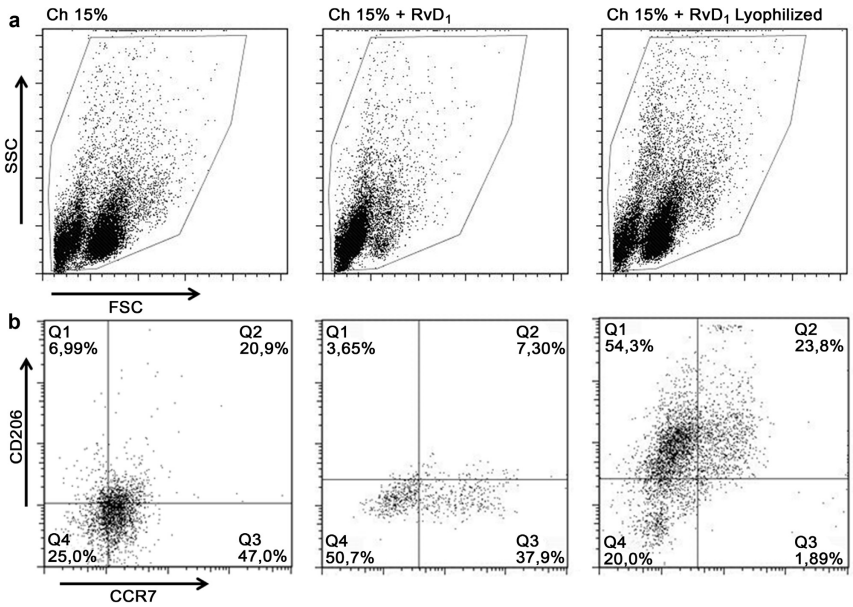



**Figure 3 | Representative dot plots of the expression of cell surface markers on macrophages of inflammatory exudates retrieved from mouse air-pouches 4 days after biomaterial implantation.** (a)

aspect ratio; (b) expression of F4/80, CD206 and CCR7 antigens. Gates were drawn from isotype control staining. (FSC: forward scatter; SSC: side scatter).

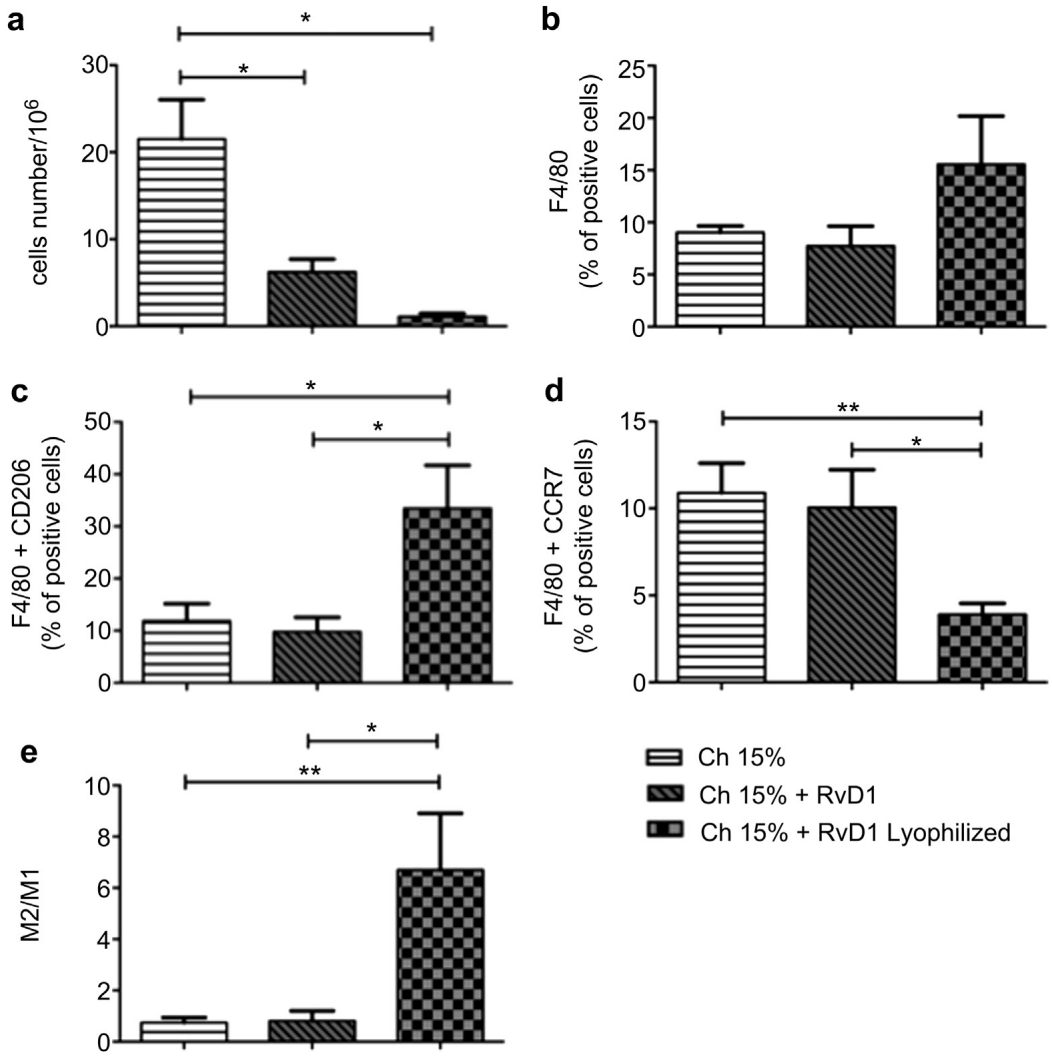



**Figure 4 | Characterization by flow cytometry of the inflammatory exudates recovered from mouse air-pouches 4 days after implantation.** Results are presented as average ± s.e.m. (a) Total number of cells recruited to the air-pouches. (b) Percentage of F4/80+ cells. (c) Cells expressing both F4/80+ and CD206+. (d) Cells expressing both F4/80+ and CCR7+. (e) Ratio of M2/M1 cells. (Statistically significant differences: *$p < 0.05$; **$p < 0.01$).

### *3.4. Cytokine production*

The detected cytokines are listed in Fig. 5a together with an image of the membranes obtained, and in Fig. 5b the quantification of the results is presented. We concluded that the implantation of scaffolds with RvD1 subjected to lyophilization caused a general decrease in pro-inflammatory cytokines, in comparison with the effect of Ch alone or Ch with RvD1 not submitted to lyophilization. In fact, this material induced a clear decrease (i) in pro-inflammatory cytokines IL-1$\alpha$ and IL-1$\beta$; (ii) in CD54 concentrations, an

intercellular adhesion molecule that is continuously pre- sent in low concentration on the membranes of leukocytes, a molecule that can be induced by IL-1; (iii) in macrophage inflammatory proteins MIP-1a, MIP-1b and MIP-2; (iv) in the cytokines IL-6 and IL-16 that were not detectable. These results are indicative of an M2 macrophage response which is in agreement with the results obtained by the flow cytometry analysis presented above.

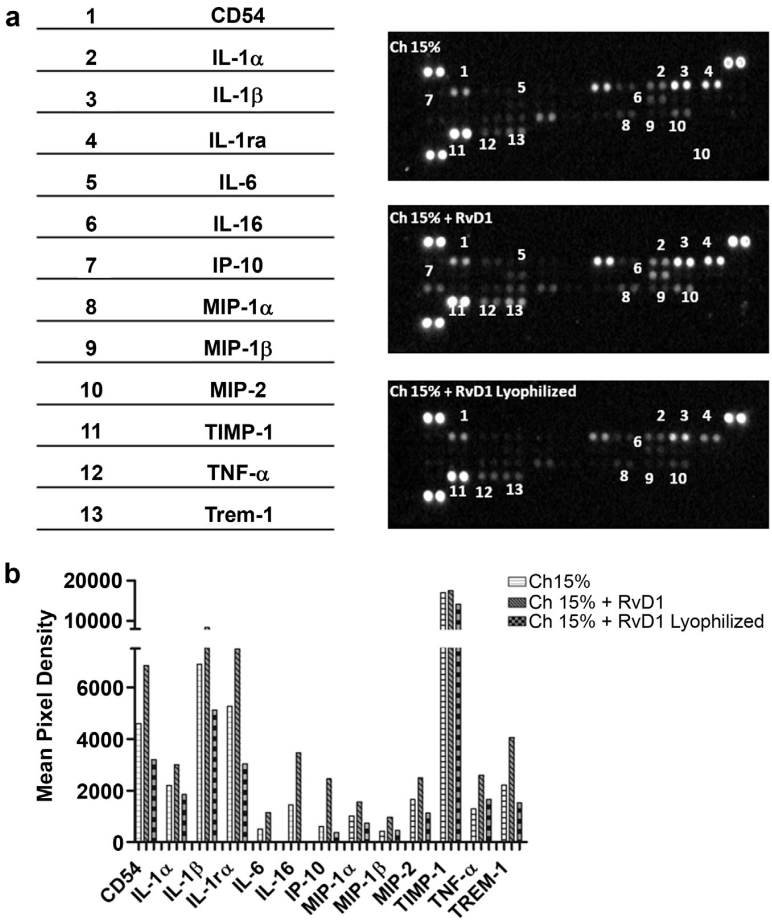



**Figure 5 | Evaluation of cytokine production by inflammatory cells on the recovered exudates 4 days after implantation using a cytokine array.** Quantification of the results was generated by quantifying the mean spot pixel density from the array using image software analyses (ImageLab; BioRad). The densities of signals were normalized with the background. (a) Images of the array membranes obtained for each experimental group and a table with the detected cytokines. (b) Quantification of the detected cytokines, values are expressed as average of two dots. (CD: cluster designation; IL: interleukin; IP: Interferon gamma-induced protein; MIP: macrophage inflammatory protein; TNF: tumour necrosis factor; TREM: triggering receptor expression on myeloid cells).

### *3.5. Histological analysis*

The implanted Ch scaffolds were analyzed by histology, 4 days after implantation, to assess their infiltration by inflammatory cells. The tissue sections were stained using Masson trichrome and the results are presented in Fig. 6. In the lower magnification

images (a-c) a general view of the inflammatory cells surrounding and colonizing the implanted scaffolds can be observed, whereas in the higher magnification images (d-f) a more detailed view of the inflammatory cells that are infiltrating the Ch scaffolds is presented. In the presence of either Ch alone (Fig. 6a and d) or Ch with RvD1 non-lyophilised (Fig. 6b and e), high numbers of inflammatory cells were seen surrounding and infiltrating the implanted scaffolds. In samples of Ch with RvD1 subjected to lyophilisation (Fig. 6c and f) there are lower numbers of inflammatory cells both surrounding and colonizing the implanted material, indicating that a less severe inflammatory cell infiltration was triggered by the developed biomaterial.

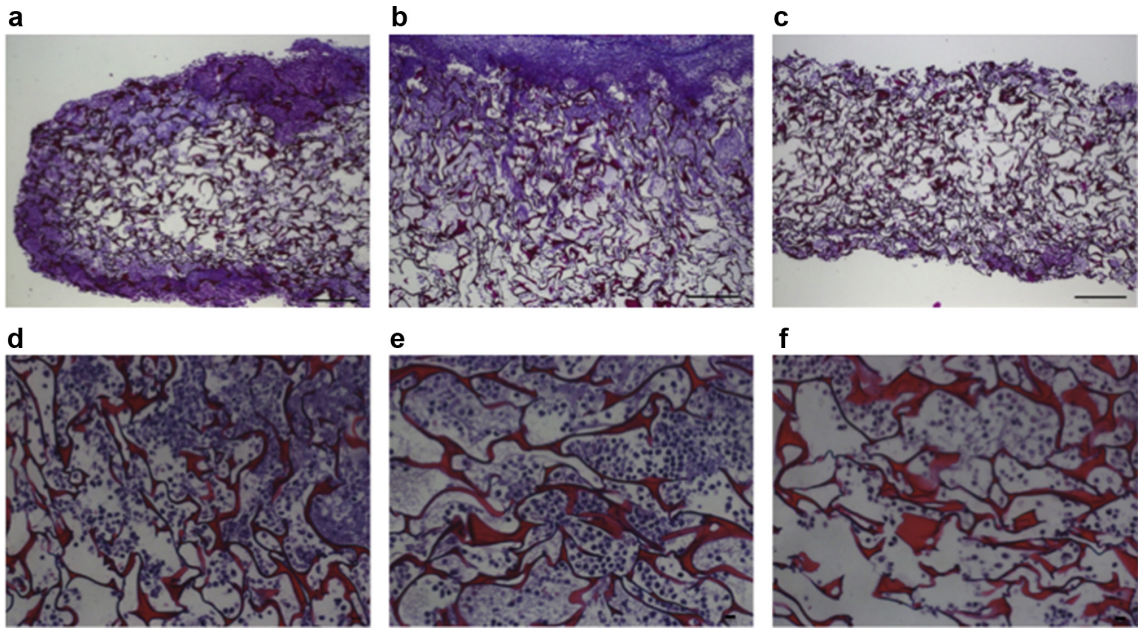



**Figure 6 |** Histological images of the Ch scaffolds 4 days after implantation in the mouse air-pouches. (a-f) Masson trichrome stained sections showing inflammatory cells around and within the implanted scaffolds. (a, d) Ch 15%; (b, e) Ch 15% + RvD1; (c, f) Ch 15% + RvD1 Lyophilized. (a, b, c: magnification10x; Scale bar =200μ mm); (d, e, f: magnification 40x; Scale bar = 10 μm).

## 4. DISCUSSION

A key factor for the long-term survival and function of biomaterials is not to elicit a detrimental immune response in the host. As biomaterials can have profound impacts on the host immune response, efforts have been made to design biomaterials that are able to lead to desired immunological outcomes and thus favor the healing process [9]. Host macrophage response is an essential component of the tissue remodeling process that follows the implantation of biomaterials [18]. Macrophages have heterogeneous phenotypes ranging from M1 (classically activated, pro-inflammatory) to M2 (anti-inflammatory, homoeostatic, wound- healing) and the remarkable plasticity makes them an interesting target for immunomodulation, since transition to an M2 phenotype is associated with improved tissue repair and regeneration [9,18].

The localized delivery of immunomodulatory factors has emerged as a strategy for controlling the local immune response to implanted biomaterials as an attempt to enhance and accelerate implant-tissue integration [1,19-23]. In our research, we aimed at developing a material capable of eliciting an appropriate immune response through the polarization of macrophages to an M2 anti-inflammatory, pro-tissue healing phenotype which is considered as a promising strategy to mitigate potential adverse effects of biomaterial-induced inflammation [24].

In previous investigations we have concluded that the injection of resolvin D1 (RvD1) every 12 h during 3 days in air pouches where Ch scaffolds are implanted caused a shift in the inflammatory response to the implanted materials through the polarization of macrophages towards an M2 phenotype [6]. In the present study we have incorporated RvD1 in 3D porous Ch scaffolds, previous to implantation, allowing the continuous release of RvD1 from the implanted Ch scaffolds. Using this strategy, we were able to develop a new functional biomaterial capable of triggering an appropriate immune response in the host. The presence of RvD1 induces a more pro-wound healing state at the implant location.

We have used the inflammatory air-pouch model of inflammation. This model involves the formation of a sterile subcutaneous cavity that can be used to insert a biomaterial and to study the inflammatory reaction caused by the biomaterial, providing a localized environment in which to study cell trafficking and the inflammatory response through the infiltration of inflammatory cells and the production of cytokines/chemokines [25,26]. The air pouch model has also been extensively used by other research groups for the study of various types of inflammation and inflammatory processes. This model has distinct

advantages over other models of inflammation because of the technical ease of the procedure and the ability to perform biochemical analysis of both the exudate and inflammatory cells together with the histological analysis of the air-pouch lining [27].

Resolvins are molecules that were first identified in self-resolving murine exudates, using the murine air pouch model of inflammation, and have been suggested to ensure restoration of tissue homoeostasis and prevention of fibrosis after the acute inflammatory response [28]. Resolvins are currently under investigation for different applications. For instance, it has been reported that RvD1 is a potent inhibitor of cigarette smoke-induced pro-inflammatory signalling in human lung cells *in vitro* and that it has potent anti-inflammatory properties in a mouse model of acute cigarette smoke-induced lung inflammation [29]. It was also demonstrated that RvD1 causes a functional switch in adipose tissue macrophage polarization towards an M2-like phenotype in obese mice [30]. Resolvins also reduce inflammation-induced mechanical hypersensitivity in the carrageenan model of peripheral inflammation in the rat through its spinal administration [7].

We have used Ch scaffolds with a DA of 15% because they are particularly adequate to study dampening of inflammation. In our previous investigations we concluded that these scaffolds caused an intense inflammatory response mediated by M1 pro-inflammatory macrophages, with large numbers of M1 macrophages present in the inflammatory exudates together with high levels of pro-inflammatory cytokines [15]. We have thus selected this material so that we can test how it is possible to shift the macrophage phenotypic response using this new strategy. Also, in agreement with previous studies, we have chosen 4 days of Ch implantation to recover the inflammatory exudates, to analyse them by flow cytometry, to assess the levels of cytokines using a cytokine array, and to remove the implants for histological analysis. The implantation of Ch scaffolds subjected to lyophilisation after RvD1 incorporation (the new strategy herein presented) caused a macrophage M2 reparative response, in contrast with a M1 pro-inflammatory response observed with the two other experimental groups used as controls. Through the analyses of the inflammatory exudates by flow cytometry we detected that the developed material induced a significant decrease in the number of recruited inflammatory cells to the implant site, together with a higher percentage of CD206+ cells (M2 macrophages) and a lower percentage of CCR7+ cells (M1 macrophages). Using a cytokine array, we have investigated the levels of some cytokines in the retrieved inflammatory exudates and found a general decrease in several pro-inflammatory cytokines (such as IL-1$\alpha$, IL-1$\beta$ and IL-6). Histology of explanted scaffolds revealed a decrease of inflammatory cells around and within the implanted scaffolds. These results confirmed that we were able to design an

immunomodulatory biomaterial that once implanted caused a shift in the host response fostering faster tissue repair and regeneration, since higher ratios of M2:M1 macrophages are associated with better remodelling outcomes [12]. We were not able to detect in the recovered inflammatory exudates the anti-inflammatory cytokines IL-4 and IL-10 in any of the experimental groups. In previous investigations we were not able to detect the presence of anti-inflammatory cytokines in the collected exudates even using ELISA kits with higher sensitivity [15].

We have incorporated RvD1 in the Ch scaffolds through an embedding technique immediately followed by lyophilisation; to assess the effect of lyophilisation after RvD1 incorporation a control group not submitted to lyophilisation after RvD1 incorporation was included in the study. We concluded that lyophilisation after RvD1 incorporation delayed its release, and that, after the release was initiated, higher concentrations of RvD1 were detected, when compared with control groups. Our results suggest that this delay in RvD1 release together with the subsequent continuous release of this lipid mediator is of great importance to induce the shift to an M2 macrophage phenotype. It has been reported that lyophilisation is a common procedure to increase the long-term stability of pharmaceutical formulations [31] and is also reported to be a lipid stabilizer [32]. As pro-resolution lipid mediators are rapidly degraded [33,34], the effect of lyophilisation after the incorporation of RvD1 in the scaffolds seems to be critical for its successful application. Also, the protective effect of the lyophilisation in RvD1 may explain why this pro-resolution mediator is released in lower concentrations by the scaffolds not submitted to lyophilisation after RvD1 incorporation. It is possible that the in this case RvD1 undergoes a fast degradation and consequently is not detected in the release assay.

The engineering of materials that can modulate the immune system is an emerging field that is progressing alongside immunology, furthermore it is recognized the potential of biomaterials that are able of modulate immune cell function and thus eliciting appropriate immune responses [35]. Taken together, our results demonstrate that we were able to develop a process of immunomodulation by incorporation of bioactive molecules. Using this strategy, we were able to create a simple and inexpensive method to deliver an anti-inflammatory mediator that allowed the *in vivo* modulation of the inflammatory response, with expected beneficial effects upon tissue remodelling.

To the best of our knowledge this is the first *in vivo* study using Ch scaffolds incorporated with RvD1 as an immunomodulatory biomaterial that allowed the modulation of macrophage polarization towards an M2 anti-inflammatory, pro-tissue healing phenotype. This strategy

provides control of macrophage phenotype and may meet with great success in regenerative medicine.

## 5. CONCLUSIONS

We have developed a new strategy to produce an immuno- modulatory biomaterial that is able of guiding the inflammatory response through a M2 macrophage reparative response. We have incorporated a pro-resolution lipid mediator, RvD1, in Ch scaffolds and afterwards phenotypic and functional polarization of macrophages in response to the implanted scaffolds was assessed according to their cell surface markers and their cytokine expression profiles. Our data demonstrated that with the developed material we induced (i) a significant decrease in the number of recruited inflammatory cells to the implant site, together with a higher percentage of CD206+ cells (M2 macrophages) and a lower percentage of CCR7+ cells (M1 macrophages); (ii) a general decrease in several pro-inflammatory cytokines; (iii) a decrease in inflammatory cells observed around and within the implanted scaffolds. These results document that we were able to experimentally shift the inflammatory response to the implanted materials, being thus a promising strategy to be used in regenerative medicine applications since the manipulation of macrophage effector mechanisms will promote constructive tissue remodeling.

## *ACKNOWLEDGMENTS*

This work was financed by FEDER Funds through the Programa Operacional Factores de Competitividade e COMPETE and Portuguese funds through FCT e Fundação para a Ciência e Tecnologia in the framework of project PTDC/SAU-BMA/113030/2009. I.F. Amaral is funded by QREN through program ON.2, in the framework of Project “NORTE-07-0124-FEDER-000005 - Project on Biomedical Engineering for Regenerative Therapies and Cancer”.

**REFERENCES**

[1] Gower RM, Boehler RM, Azarin SM, Ricci CF, Leonard JN, Shea LD. Modulation of leukocyte infiltration and phenotype in microporous tissue engineering scaffolds via vector induced IL-10 expression. Biomaterials 2014;35:2024-31.

[2] Eming SA, Krieg T, Davidson JM. Inflammation in wound repair: molecular and cellular mechanisms. J Invest Dermatol 2007;127:514-25.

[3] Li YS, Dalli J, Chiang N, Baron RM, Quintana C, Serhan CN. Plasticity of leukocytic exudates in resolving acute inflammation is regulated by MicroRNA and proresolving mediators. Immunity 2013;39:885-98.

[4] Freire MO, Van Dyke TE. Natural resolution of inflammation. Periodontol 2000 2013;63:149-64.

[5] Widgerow AD. Cellular resolution of inflammationecatabasis. Wound Repair Regen 2012;20:2-7.

[6] Vasconcelos DP, Costa Madalena, Amaral IF, Barbosa MA, Águas AP, Barbosa JN. Modulation of the inflammatory response to chitosan through M2 macrophage polarization using pro-resolution mediators. Biomaterials 2015;37:116-23.

[7] Abdelmoaty S, Wigerblad G, Bas DB, Codeluppi S, Fernandez-Zafra T, El-Awady el S, et al. Spinal actions of lipoxin A4 and 17(R)-resolvin D1 attenuate inflammation-induced mechanical hypersensitivity and spinal TNF release. PloS One 2013;8:e755-43.

[8] Franz S, Allenstein F, Kajahn J, Forstreuter I, Hintze V, Moller S, et al. Artificial extracellular matrices composed of collagen I and high-sulfated hyaluronan promote phenotypic and functional modulation of human pro-inflammatory M1 macrophages. Acta Biomater 2013;9:5621-9.

[9] Franz S, Rammelt S, Scharnweber D, Simon JC. Immune responses to implants - a review of the implications for the design of immunomodulatory bio- materials. Biomaterials 2011;32:6692-709.

[10] Kou PM, Babensee JE. Macrophage and dendritic cell phenotypic diversity in the context of biomaterials. J Biomed Mater Res A 2011;96:239-60.

[11] Mokarram N, Merchant A, Mukhatyar V, Patel G, Bellamkonda RV. Effect of modulating macrophage phenotype on peripheral nerve repair. Biomaterials 2012;33:8793-801.

[12] Brown BN, Londono R, Tottey S, Zhang L, Kukla KA, Wolf MT, et al. Macro- phage phenotype as a predictor of constructive remodeling following the implantation of biologically derived surgical mesh materials. Acta Biomater 2012;8:978-87.

[13] Vachoud L, Zydowicz N, Domard A. Formation and characterisation of a physical chitin gel. Carbohydr Res 1997;302:169-77.

[14] Barbosa JN, Amaral IF, Águas AP, Barbosa MA. Evaluation of the effect of the degree of acetylation on the inflammatory response to 3D porous chitosan scaffolds. J Biomed Mater Res A 2010;93:20-8.

[15] Vasconcelos DP, Fonseca AC, Costa M, Amaral IF, Barbosa MA, Águas AP, et al. Macrophage polarization following chitosan implantation. Biomaterials 2013;34:9952-9.

[16] Sedwick AD, Sin YM, Edwards JC, Willoughby DA. Increased inflammatory reactivity in newly formed lining tissue. J Pathol 1983;141:483-95.

[17] Castro AG, Esaguy N, Macedo PM, Águas AP, Silva MT. Live but not heat-killed mycobacteria cause rapid chemotaxis of large numbers of eosinophils *in vivo* and are ingested by the attracted granulocytes. Infect Immun 1991;59: 3009-14.

[18] Brown BN, Badylak SF. Expanded applications, shifting paradigms and an improved understanding of host-biomaterial interactions. Acta Biomater 2013;9:4948-55.

[19] Couto DS, Perez-Breva L, Saraiva P, Cooney CL. Lessons from innovation in drug-device combination products. Adv Drug Deliv Rev 2012;64:69-77.

[20] Wu P, Grainger DW. Drug/device combinations for local drug therapies and infection prophylaxis. Biomaterials 2006;27:2450-67.

[21] Zilberman M. Active implants and scaffolds for tissue regeneration. BerlinHeidelberg: Springer-Verlag; 2011.

[22] Lewis A. Drug device combination products: delivery technologies and applications. Cambridge: Woodhead Publishing Limited; 2010.

[23] Domb AJ, Khan W. Focal controlled drug delivery. Springer; 2014.

[24] Brown BN, Ratner BD, Goodman SB, Amar S, Badylak SF. Macrophage polarization: an opportunity for improved outcomes in and regenerative medicine.
Biomaterials 2012;33:3792-802.

[25] Wooley PH, Morren R, Andary J, Sud SA, Yang SY, Mayton L, et al. Inflammatory responses to orthopaedic biomaterials in the murine air pouch. Biomaterials 2002;23:517-26.

[26] Hooper KA, Nickolas TL, Yurkow EJ, Kohn J, Laskin DL. Characterization of the inflammatory response to biomaterials using a rodent air pouch model. J Biomed Mater Res 2000;50:365-74.

[27] Winyard PG, Willoughby DA. Methods in molecular biology. Inflammation protocols. New Jersey: Humana Press; 2003.

[28] Serhan CN, Clish CB, Brannon J, Colgan SP, Chiang N, Gronert K. Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2-nonsteroidal antiinflammatory drugs and transcellular processing. J Exp Med 2000;192: 1197-204.

[29] Hsiao HM, Sapinoro RE, Thatcher TH, Croasdell A, Levy EP, Fulton RA, et al. A novel anti-inflammatory and pro-resolving role for resolvin D1 in acute cigarette smoke-induced lung inflammation. PloS One 2013;8. e58258.

[30] Titos E, Rius B, Gonzalez-Periz A, Lopez-Vicario C, Moran-Salvador E, Martinez-Clemente M, et al. Resolvin D1 and its precursor docosahexaenoic acid promote resolution of adipose tissue inflammation by eliciting macrophage polarization toward an M2-like phenotype. J Immunol 2011;187:5408-18.

[31] Di Tommaso C, Como C, Gurny R, Moller M. Investigations on the lyophilisation of MPEG-hexPLA micelle based pharmaceutical formulations. Eur J Pharm Sci 2010;40:38-47.

[32] Howard MD, Lu XL, Jay M, Dziubla TD. Optimization of the lyophilization process for long-term stability of solid-lipid nanoparticles. Drug Dev Ind Pharm 2012;38:1270-9.

[33] Maderna P, Godson C. Lipoxins: resolutionary road. Br J Pharmacol 2009;158: 947-59.

[34] Mitchell S, Thomas G, Harvey K, Cottell D, Reville K, Berlasconi G, et al. Lip- oxins, aspirin-triggered epi-lipoxins, lipoxin stable analogues, and the resolution of inflammation: stimulation of macrophage phagocytosis of apoptotic neutrophils in vivo. J Am Soc Nephrol 2002;13:2497-507.

[35] Hubbell JA, Thomas SN, Swartz MA. Materials engineering for immunomodulation. Nature 2009;462:449-60.

# CHAPTER V

## ORIGINAL RESEARCH WORK

### IMMUNOMODULATORY BIOMATERIALS AND BONE REPAIR

#### ARTICLE III

"Chitosan porous 3D scaffolds embedded with resolving D1 to improve *in vivo* bone healing." Journal of Biomedical Materials Research Part A (2018).

## Chitosan porous 3D scaffolds embedded with resolvin D1 to improve *in vivo* bone healing

Daniela P. Vasconcelos,[1,2,3] Madalena Costa,[3,4] Nuno Neves,[1,2,5,6] José H. Teixeira,[1,2,3] Daniel M. Vasconcelos,[1,2,3] Susana G. Santos,[1,2] Artur P. Águas,[3,4] Mário A. Barbosa,[1,2,3] Judite N. Barbosa[1,2,3]

[1]i3S – Instituto de Inovaçãoo e Investigaçãoo em Saúde, Universidade do Porto, Porto 4200-125, Portugal
[2]INEB – Instituto de Engenharia Biomédica, Porto 4200-125, Portugal
[3]ICBAS – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto 4050-313, Portugal
[4]UMIB – Unit for Multidisciplinary Biomedical Research of ICBAS
[5]FMUP – Faculdade de Medicina, Universidade do Porto, Porto 4200, Portugal
[6]Spine Group, Orthopedic Department, Hospital de São João, Porto 4200-319, Portugal

**ABSTRACT**

The aim of this study was to investigate the effect chitosan (Ch) porous 3D scaffolds embedded with resolvin D1 (RvD1), an endogenous pro-resolving lipid mediator, on bone tissue healing. These scaffolds previous developed by us have demonstrated to have immunomodulatory properties namely in the modulation of the macrophage inflammatory phenotypic profile in an *in vivo* model of inflammation. Herein, results obtained in an *in vivo* rat femoral defect model demonstrated that two months after Ch+RvD1 scaffolds implantation, an increase in new bone formation, in bone trabecular thickness, and in Coll type I and Coll I/ Coll III ratio were observed. These results suggest that Ch scaffolds embedded with RvD1 were able to lead to the formation of new bone with improvement of trabecular thickness. This study shows that the presence of RvD1 in the acute phase of the inflammatory response to the implanted biomaterial had a positive role in the subsequent bone tissue repair, thus demonstrating the importance of innovative approaches for the control of immune responses to biomedical implants in the design of advanced strategies for regenerative medicine.

***KEYWORDS***

Biomaterials, chitosan healing, resolving D1, Immunomodulation

## 1. INTRODUCTION

Regenerative medicine is an emerging field with great potential and vast applications. There are two main strategies for tissue repair: cell-based therapies and the promotion of endogenous repair. Recent findings have highlighted new and often unexpected roles for immune cells in promoting a permissive local environment for effective cell replacement and restoration of tissue integrity [1]. Biomaterial scaffolds are central to many tissue repair strategies as they create a space for tissue growth and provide a support for cell adhesion and migration. However, biomaterial implantation causes injury resulting in inflammatory response which can impair its integration and the subsequent tissue repair [2]. As biomaterials can have profound impacts on the host immune response the concept of designing immunomodulatory biomaterials, able to trigger desired immunological outcomes, has emerged [3]. Bone tissue repair is currently under extensive investigation in tissue engineering and regenerative medicine research. Bone fracture is a common and increasing medical affliction that results from both traumatic injury and disease-related bone fragility [4,5]. Even when adequate bone repair is achieved within the expected time frames, treating fractures is costly. The most common cause of bone fragility is osteoporosis, and the socioeconomic burden of osteoporotic fractures alone is an increasing global problem for healthcare systems. Clearly, benefit can be gained from improved understanding of bone tissue repair [6]. The inflammatory response is an inevitable consequence of tissue injury, and its resolution is essential to return to tissue homeostasis [7]. A rapid resolution of the inflammatory response and transition to the repair phase is crucial to a favorable outcome of the tissue under regeneration [8]. The resolution of the inflammatory response is an active process controlled by endogenous mediators such as resolvins, lipoxins, protectins, and maresins that have selective actions on inflammatory cells and actively drive the termination of inflammation [9,10]. Resolvins are generated from docosahexaenoic acid [11]. It has been demonstrated that RvD1 controls neutrophils recruitment [12] and modulates macrophages toward a phenotype more prone to a healing response [13]. Resolvins also improve wound healing and reduce the time of wound closure [14]. Biomaterial-based scaffolds such as polymeric porous blocks have gained some success in the field of bone regeneration, with data providing that these materials are biocompatible and enable the incorporation of bioactive molecules and also the regulation of their kinetics release [15]. Chitosan is a natural polymer chemically constituted by $\beta$ (1–4) linked D-glucosamine residues with N-acetyl-glucosamine side chains. Chitosan has been used for several biomedical applications, namely for bone tissue engineering studies [16-18]. We have previously reported the development of an immunomodulatory strategy that results from the incorporation of RvD1, in a porous 3D Ch scaffold (Ch + RvD1). Using this strategy, we

were able to trigger *in vivo* a shift in the macrophage response toward a M2 reparative response using a rodent air-pouch model of inflammation [19]. In the present study, we have investigated the ability of this novel immunomodulatory strategy to improve bone tissue healing, using a rat femoral bone defect model.

## 2. MATERIALS & METHODS

### *2.1. Preparation of 3D Ch Scaffolds*

Squid pen Ch (reference 114, Batch No. S4; DA ~2%) was supplied by Mahtani Chitosan Pvt. Ltd., purified and prepared with a degree of acetylation (DA) of 15%, as previously described [13,20]. Briefly, 3D porous scaffolds were prepared from degassed 2% w/v Ch solutions in 0.2M acetic acid via thermally induced phase separation (-20º) and subsequent sublimation of the ice crystals. Following lyophilization (-80ºC; 0.2mbar; 24 h), the resultant scaffolds were cut in a cylinder shape with 4 mm in diameter and a height of 5 mm. The scaffolds were then submitted to sterilization in an ethanol series: immersed under vacuum in absolute ethanol (B Braun) for 1 h and then sequentially in 70% (v/v) for 30 min, 50% (v/v) and 25% (v/v) ethanol for 10 min each. Afterwards, Ch scaffolds were washed twice with ultrapure water (Milli Q) for 10 min. Scaffolds were then frozen (-20ºC; 24 h) and lyophilized again (-80ºC; 0,2mbar; 48 h) under sterile conditions (scaffolds were placed in 96 well plates at the freeze dryer with a filter membrane of 0,22 μm).

### *2.2. Incorporation of RvD1 in the Ch scaffolds*

The incorporation of RvD1 (Cayman Chemical, Ann Arbor, Michigan) in the Ch scaffolds is described in detail elsewhere [19]. Briefly, RvD1 was incorporated in Ch scaffolds by an embedding technique under sterile conditions. An ethanolic solution of RvD1 (Cayman Chemical) with a concentration of 3.33 ng/μL was prepared according to the provider instructions. A volume of 30 μL of RvD1 solution was added drop-wise with a micropipette into the scaffold which was then lyophilized again (-80ºC; 0,2mbar; 24 h). Chitosan scaffolds without RvD1 were used as a control group, being these scaffolds submitted to the same number of lyophilizations.

### *2.3. Animal Model*

We have used a rat femoral defect model based on the study of Le Guehennec *et al* [21] previously adapted by our research group [16]. Three-month-old male Wistar rats purchased from Charles River Laboratories Spain, (Barcelona, Spain) (n=5 per group) were used. Animals were anesthetized for surgery through isoflurane inhalation. The knees were shaved and disinfected, an incision was made in the skin and muscles were retracted. After lateral knee arthrotomy, a cylindrical defect with a diameter of 3 mm and a depth of approximately 4 mm was created using a surgical drill in the anterolateral wall of the lateral condyle of right femur. Animal care and analgesics (subcutaneous injection of Buprex-Buprenorfina, 0.05 mg/$Kg^{-1}$) were provided post- surgery. Each animal was implanted with Ch + RvD1 or Ch alone in the right femur defect. All animals were sacrificed 2 months after

implantation. Non-operated animals were used as additional controls. Experimental protocols were performed in accordance with guidelines approved by the Ethics Committee and the Portuguese Official Authority on Animal Welfare and Experimentation.

### *2.4. Bone histological analysis*

Cleaned femurs were fixed, decalcified in 0.25M EDTA (Sigma) (pH 7.3) for 7 weeks at room temperature with agitation and dehydrated in an ethanol series. Samples were embedded in paraffin blocks and eight serial sections of each femur, with 30 μm between sections (seven slices per section with 3 μm-thickness for each slice) were prepared. The histological sections were then stained with both Masson's trichrome and picrosirius red, according to standard protocols, being afterwards observed and digitalized using a stereomicroscope (SZX10, Olympus) or an inverted microscope (Axiovert 200M, Zeiss).

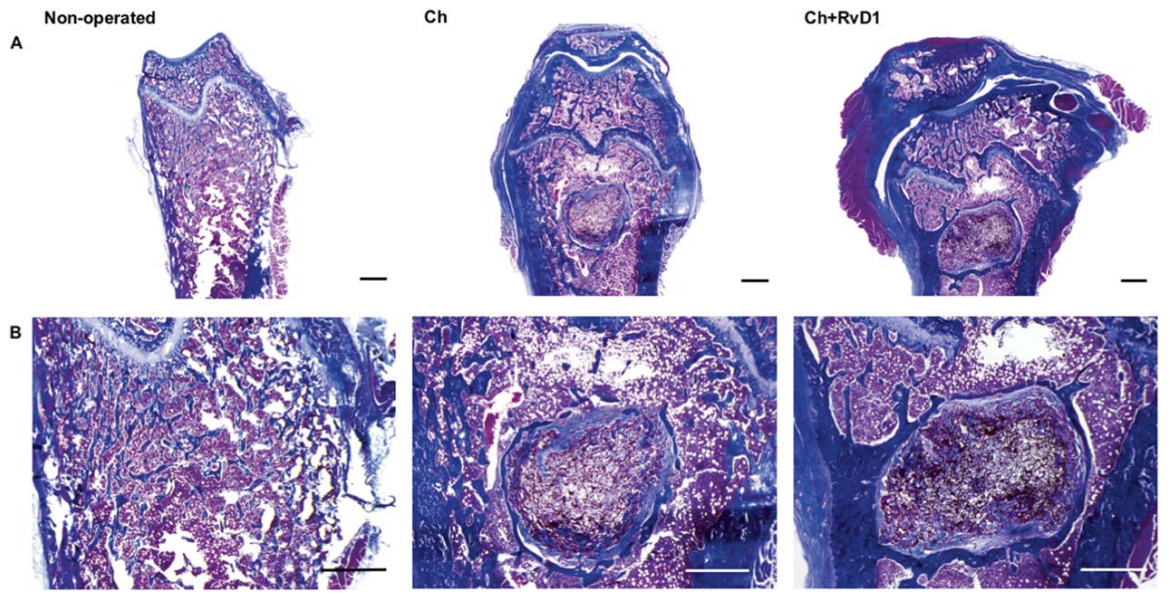



**Figure 1 |** Histological sections stained with Masson's trichrome of the femur of non-operated and with critical size bone defects created in the anterolateral wall of the lateral condyle and subsequently implanted with Ch or Ch+RvD1 scaffolds. Histological evaluation was performed 2 months after implantation. (A) Global view of the defects with implanted scaffolds (Scale bar = 1 mm) and (B) detailed view of the implanted area (scale bar = 1 mm).

### *2.5. Collagen quantification*

The picrosirius red staining was used as a method for the quantification of Coll birefringent fibers to assess the structural changes in the newly formed bone, as described elsewhere [22,23]. Serial sections (three slices of different serial sections for each femur) with 3 μm thickness, were stained with picrosirius red and visualized through a polarized lens coupled to a binocular inverted microscope (Zeiss, Axiovert 200M) under 20X magnification, being the images acquired using MosaiX software. All images were captured with the same parameters (light intensity and angle of the polarizing lens). Image J software (version 2.2) was used to delimit the region of interest (ROI, defect and scaffold area) and for the quantification of the birefringence brightness. For that, images were binarized for definition of green, blue and red color spectra, and the quantity of each color pixels corresponding to the total area were measured. The Coll I/Coll III ratios were calculated as fold change relative to the non-operated animals. Values of 3 sections from each animal were calculated.

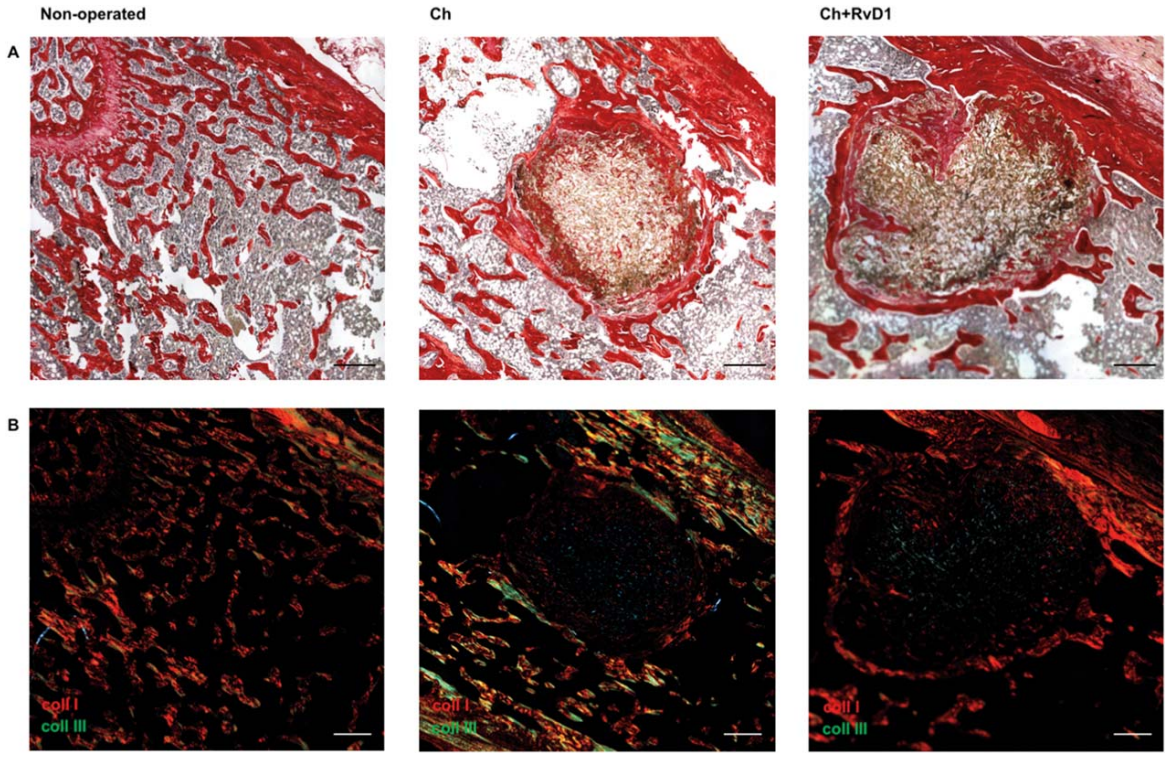



**Figure 2 |** Histological sections of picrosirius red staining of the non-operated animals and of critical size bone defects of animals with implanted Ch or Ch + RvD1 scaffolds, visualized upon polarized and conventional light to identify Coll fibers types. Histological evaluation was performed 2 months after implantation. (A) Transmitted light. (B) Polarized light. Green birefringence colour indicates thin fiber and red colours in the birefringence analysis indicate thick Coll fibers. Original magnification 20x. Bar= 500 μm.

### *2.6. Micro – computed tomography*

Bone defects (n=5) were fixed in 10% formalin. Microcomputed tomography (μCT) was used to evaluate new bone formation within the defect region. The structure of the samples was acquired by X-ray and then reconstructed and analyzed. The hard tissues (corresponding to bone) were achieved through the volume of interest (VOI), consisting of a stack of ROIs drawn over 150 cross sections. For morphometric analysis, the following structural parameters were calculated over each VOI: bone volume fraction (BV/TV) and trabecular thickness (Tb.Th). Data acquisition was performed in a SkyScan 1272 scanner with a pixel size of 7.4 μm. Approximately 600 projections were acquired over a rotation range of 180º, with a rotation step of 0.45º. Datasets were reconstructed using standardized cone-beam reconstruction software (NRecon v. 1.6.10.2, SkyScan). The output format for each sample was bit-map images. The set of images was orientated with DataViewer (v. 1.5.2.4, SkyScan) to obtain all samples in the same axis. A representative dataset of the slices was segmented into binary images with a dynamic threshold of 70–255 for hard tissue analysis. Afterwards, the binary images were used for morphometric analysis (CT Analyser, v. 1.16.4.1, SkyScan).

### *2.7. Statistical analysis*

Statistical analysis was performed using the non-parametric test Mann–Whitney to compare data from two different groups, with Prism v6 for Mac OS X. A value of $p<0.05$ was considered statistically significant: *$p<0.05$. Data are presented showing individual groups and respective mean ± SD.

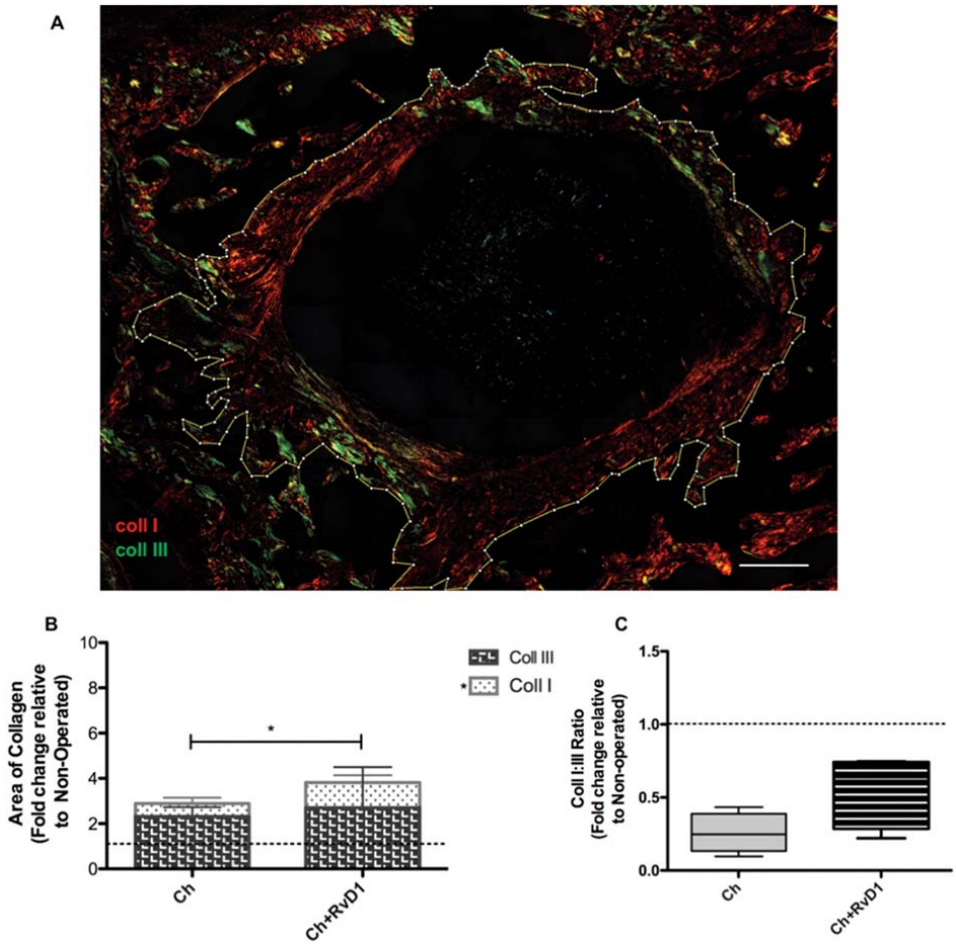



**Figure 3 |** Quantification of birefringent Coll fibers. Intensity of birefringence was measured with the image-analysis software (Image J, v. 2.0.). (A) Region of interest (defect and scaffold area). (B) Total area of Coll fibers from each birefringence colour (%). (C) Coll I/Coll III ratio. Results are presented as the mean (±SEM) of area of Coll fibers (%) for each colour in the birefringence analysis. (Statistically significant differences: *$p < 0.05$).

## 3. RESULTS

### *3.1. Histological analysis and quantification of birefringent collagen fibers*

Bone histological analysis was performed on the right femurs of the animals of all experimental groups. Figure 1 presents Masson's trichrome stained sections of a general view of the femurs (A) and the detailed view of the implanted area (B). For both experimental groups (Ch or Ch+RvD1) a layer of newly formed bone was observed delimiting the defect area, being thicker in the case of the animals implanted with Ch+RvD1 scaffolds. We have also investigated whether the formation of Coll fibers was affected by the presence of RvD1. For that, we used the picrosirius-polarization method with analysis of birefringent fibers that allows the quantification of the Coll fibers. Figure 2 documents images of picrosirius red-stained sections acquired using MosaiX software and the quantification of birefringent fibers. Figure 2(A) shows sections viewed by transmitted light, whereas Figure 2(B) illustrates sections obtained using polarized lens. All images were captured with the same parameters (light intensity and angle of the polarizing lens). Collagen type I (classically called Coll fibers), that is more birefringent and thicker correspond to the red or yellow fibers whereas Coll type III (classically called reticulum fibers), that is less birefringent and thin correspond to the green fibers. Figure 3 presents the results of the Coll quantification together with an example of a defect area used for this evaluation. Animals with Ch+RvD1 scaffolds exhibited a statistically significant increase in Coll type I fibers (red fibers) when compared with animals with Ch scaffolds being the ratio of Coll I/Coll III also increased for this experimental group.

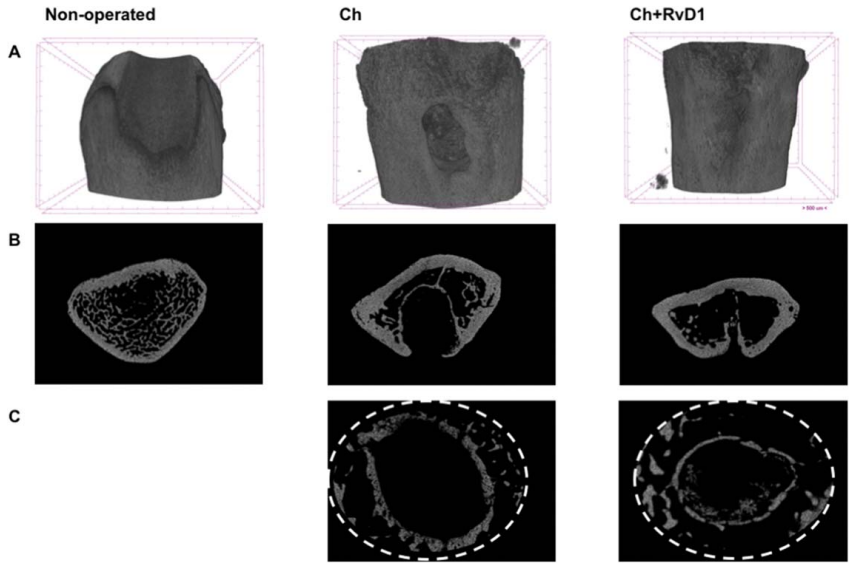



**Figure 4 |** Micro-computed tomography (μCT) images of bone defects. Samples were scanned with μCT System (Skyscan, 1272). Images were reconstructed using NRecon software. (A) Reconstructed images of the defect area. (B) Transversal section of bone defects. (C) Representative images inside the defect.

### *3.2. μCT analysis*

We have used μCT images of the different experimental groups to assess the effect of RvD1 in new bone formation. The μCT reconstruction of the defect area is showed in Figure 4(A), whereas Figure 4(B) presents transversal sections of the defects and Figure 4(C) presents detailed sections of the ROI used for the analysis. Transversal sections showed that two months after implantation the defects are still open in both experimental groups, being the defect smaller in the femurs of rats submitted to Ch+RvD1 scaffolds implantation (Fig. 4B). Interestingly, some areas with new bone formation can be observed inside the defects of this experimental group (Fig. 4C). Concerning the μCT data analysis, Figure 5A shows the quantification of bone volume fraction (bone volume/tissue volume; BV/TV); and Figure 5(B) presents the differences in the bone trabecular thickness (Tb.Th). The animals of the experimental group implanted with Ch+RvD1 scaffolds presented a statistically significant increase of the bone trabecular thickness (Fig. 5B), indicating the formation of a more robust bone when compared to the animals implanted with Ch scaffolds. In addition, animals implanted with Ch+RvD1 scaffolds showed higher bone volume than animals implanted with Ch scaffolds (Fig. 5A).

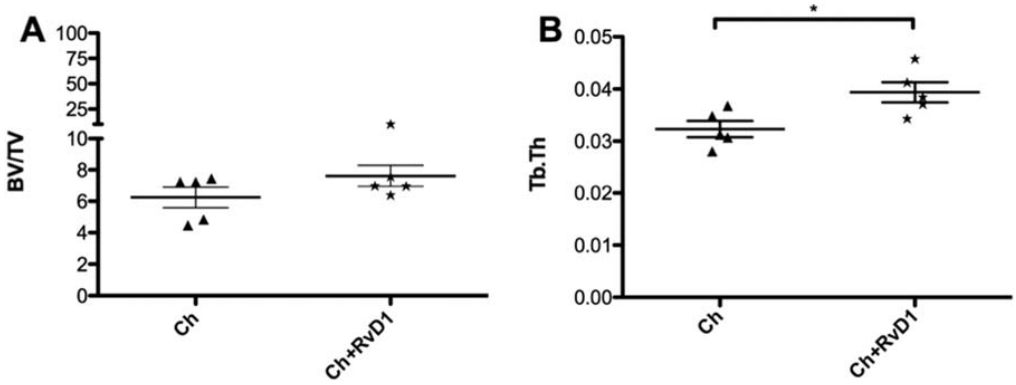



**Figure 5 |** Micro-computed tomography (μCT) analysis of bone healing process. (A) Bone volume, (B) Trabecular thickness (Tb.Th). Results are presented as average ±SEM. (Statistically significant differences: *p < 0.05).

## 4. DISCUSSION

Bone tissue engineering consists in the use of a scaffolding material that will both induce formation of bone from surrounding tissues and act as a carrier of bioactive agents [24,25]. Several strategies have been proposed to improve the efficiency of bone regeneration by making it faster, more controlled and predictable. Among them, the association between biomaterials and molecules capable of stimulating osteoblast function or that modulate inflammation or angiogenesis [26]. We report here a strategy to improve bone tissue healing through the implantation of porous 3D Ch scaffolds embedded with RvD1, an endogenous pro-resolution lipid mediator. Previous studies of ours have demonstrated that with the use of this mediator of the resolution of inflammation incorporated in Ch scaffolds we were able to develop an immunomodulatory strategy, that induces *in vivo* a shift in the macrophage phenotypic profile toward an M2 reparative phenotype during an inflammatory response to an implanted biomaterial, and thus creating an environment for prone to tissue repair [19]. In this research work we envisage the use of these Ch scaffolds embedded with RvD1 to create a local environment favourable to bone tissue repair since higher ratios of M2:M1 macrophages are associated with better remodelling outcomes [27]. Biomaterials have an important role in the implantable medical device industry improving therapeutic efficacy. The engineering of biomaterials that can modulate the immune system is today an emerging field that is progressing alongside with advances in immunology. There is solid hope on the potential of biomaterials to modulate immune cell function and thus elicit appropriate immune responses, the so-called immunomodulatory biomaterials [28]. Manipulating the immune system using biomaterials engineering may support the development of therapies that promote both systemic and local pro-regenerative immune responses, ultimately stimulating tissue repair [29]. We have used the rat femoral defect model that involves the formation of a cylindrical defect in the anterolateral wall of the lateral condyle of right femur. Based in our previous experience with this model, [16,30] and taking in consideration the time required for bone remodeling we have decided to recover the femurs two months after biomaterial implantation, being then processed for the evaluation of the bone defect area both by μCT and histological techniques. The implantation of Ch+RvD1 scaffolds induced an enhancement in bone formation, detected both by μCT and histological analysis of the defect area two months after implantation, when compared with animals implanted with Ch scaffolds alone. Histological evaluation of the different experimental groups revealed that a layer of newly formed bone was observed delimiting the defect area, being thicker in the case of the animals implanted with Ch+RvD1 scaffolds. Using μCT, a widely applied technique for the 3D study of bone microstructure, we observed a statistically significant increase in trabecular thickness (Tb.Th), that is, considered a reliable indicator of bone

strength, [31] and a key factor for the success of implant- supported rehabilitation [26]. This result is particularly relevant when considering that this healing occurred at the bone/implant interface since conventional materials fail the integration with surrounding tissues. Aseptic loosening is a typical case study of the limited integration of joint prostheses, highly motivated by adverse immune responses [32]. Furthermore, these approaches are quite relevant in diseases such as osteoporosis in which high remodeling with negative balance induces bone loss and deep alterations in the rearrangement of the microarchitecture, such as the decrease of trabecular thickness and connectivity [31]. Bone strength depends not only on the quantity of bone tissue but also on its quality. Quantity is in part evaluated by bone mineral density, whereas the quality is assessed in part by Coll [31,33]. To further analyze the new bone, we have determined the Coll that was formed through the picrosirius-polarization method and the quantification of birefringent fibers, described by Junqueira *et al*. [34,35] and previously used in the characterization Coll networks [36,37]. The evaluation of the Coll type I and type III fibers that were present in defect area implanted with scaffolds showed a significant increase in Coll type I (Coll fibers) in animals implanted with Ch+RvD1 scaffolds when compared with animals implanted with Ch scaffolds. Also, Coll I/Col III ratio showed an increase in Ch+RvD1 experimental group, although not statistically significant. These results strengthen the evidence on the new bone formation that was detected by μCT analysis, once crystals of hydroxyapatite tend to be oriented in the same direction as the Coll type I fibers, showing that these fibers are important for the mechanical properties of bone [31]. Interestingly, the loss of mechanical integrity of fibromuscular tissues is also related with a lower Coll I/Coll III ratio [37]. Although we have not found statistically significant differences in the increase bone volume fraction (BV/TV), results from trabecular thickness (Tb.Th), together with Coll quantification, show us that RvD1 induced the formation of bone with parameters associated with increased strength and quality. The role of resolvins has been studied in many pathologies [38]. For instance, in adipose tissue, RvD1 is responsible for switching macrophage polarization toward an M2-like phenotype in obese mice [39]. It was also reported in an *in vitro* study that RvD1 was able to suppress factors that are involved in osteoarthritis pathogenesis [40]. Local delivery of RvD1 leads to the resolution of inflammatory response after myocardial infarction improving ventricular function, [41] and to the enhancement of wound closure in diabetic mice by promoting macrophage polarization toward the anti-inflammatory M2 phenotype [14]. Additionally, the treatment of mice with RvD1 exposed to cigarette smoke, significantly reduced lung inflammation [42]. The use of biomaterials to locally deliver suitable levels of RvD1 constitute a promising approach to mitigate exacerbated immune responses in bone injury contexts. We hypothesize that to achieve a clear improvement of bone repair it would be necessary to accomplish a more sustained delivery of RvD1, since the injury performed in

the *in vivo* model used in this research work requires several weeks to heal. Our previous *in vitro* findings showed that RvD1 is released from Ch scaffolds mainly in 72 h [19]. Nevertheless, the presence of RvD1 will induce a more pro- wound healing state at the implant area during the early inflammatory stage of the bone healing response [4,43] being this important for the subsequent steps of bone tissue repair. We have also studied immune cell populations in blood and draining lymph nodes (data not shown) 2 months after implantation but no significant differences were observed in the immune cell populations, most likely because the changes in these cell populations can only be detected at the early time points of the biological response to implanted scaffolds. Based on the results obtained in this research work, we came to the conclusion that RvD1 has a positive effect in bone healing, thus a more in-depth understanding of the early effects of RvD1 in bone tissue healing must be now detailed dissected. To our knowledge, this is the first study to suggest that RvD1 can improve bone healing, as indicated by an increase in bone formation, trabecular thickness and Coll type I. These results may be related with the capacity of RvD1 to modulate the inflammatory response to implanted materials, showing that its presence in the acute phase of the inflammatory response to the implanted biomaterial can have a positive role in the subsequent bone repair, most likely due to the presence of macrophages exhibiting phenotypes associated with anti-inflammatory, pro-healing functions.

## *ACKNOWLEDGMENTS*

We thank Catarina L. Pereira, Catarina R. Almeida, Maria Inês Almeida, Ana Lourenço, Cláudia Machado and Andreia M. Silva for the support in the *in vivo* studies. This work was financed by: FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through FCT - Fundação para a Ciência e a Tecno- logia in the framework of the project “Institute for Research and Innovation in Health Sciences” (POCI-01-0145-FEDER-007274) and NORTE-01- 0145-FEDER-000012, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). Daniela P. Vasconcelos is funded by FCT PhD Programmes and by Programa Operacional Potencial Humano (POCH), BiotechHealth Programme (Doctoral Programme on Cellular and Molecular Biotechnology Applied to Health Sciences - reference PD/00016/2012), and through the PhD studentship PD/BD/114011/2015.

**REFERENCES**

[1] Forbes SJ, Rosenthal N. Preparing the ground for tissue regeneration: from mechanism to therapy. Nat Med 2014;20:857–869.

[2] Gower RM, Boehler RM, Azarin SM, Ricci CF, Leonard JN, Shea LD. Modulation of leukocyte infiltration and phenotype in micro- porous tissue engineering scaffolds via vector induced IL-10 expression. Biomaterials 2014;35:2024–2031.

[3] Franz S, Rammelt S, Scharnweber D, Simon JC. Immune responses to implants - a review of the implications for the design of immunomodulatory biomaterials. Biomaterials 2011;32: 6692–6709.

[4] Einhorn TA, Gerstenfeld LC. Fracture healing: mechanisms and interventions. Nat Rev Rheumatol 2015;11:45–54.

[5] Redlich K, Smolen JS. Inflammatory bone loss: pathogenesis and therapeutic intervention. Nat Rev Drug Discov 2012;11:234–250.

[6] Holroyd C, Cooper C, Dennison E. Epidemiology of osteoporosis. Best Pract Res Clin Endocrinol Metab 2008;22:671–685.

[7] Eming SA, Krieg T, Davidson JM. Inflammation in wound repair: molecular and cellular mechanisms. J Investig Dermatol 2007;127:514–525.

[8] Eming SA, Hammerschmidt M, Krieg T, Roers A. Interrelation of immunity and tissue repair or regeneration. Semin Cell Dev Biol
2009;20:517–527.

[9] Freire MO, Van Dyke TE. Natural resolution of inflammation. Periodontology 2000 2013;63:149–164.

[10] Maderna P, Godson C. Lipoxins: Resolutionary road. Br J Pharmacol 2009;158:947–959.

[11] Serhan CN, Hong S, Gronert K, Colgan SP, Devchand PR, Mirick G, Moussignac R-L. Resolvins: A family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. J Exp Med 2002; 196:1025–1037.

[12] Hong S, Gronert K, Devchand PR, Moussignac RL, Serhan CN. Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. Autacoids in anti-inflammation. J Biol Chem 2003;278:14677– 14687.

[13] Vasconcelos DP, Costa M, Amaral IF, Barbosa MA, Águas AP, Barbosa JN. Modulation of the inflammatory response to chitosan through M2 macrophage polarization using pro-resolution mediators. Biomaterials 2015;37:116–123.

[14] Tang Y, Zhang MJ, Hellmann J, Kosuri M, Bhatnagar A, Spite M. Proresolution therapy for the treatment of delayed healing of diabetic wounds. Diabetes 2013;62:618–627.

[15] Jo BS, Lee Y, Suh JS, Park YS, Lee HJ, Lee J-Y, Cho J, Lee G, Chung CP, Park KD, Park YJ. A novel calcium-accumulating pep- tide/gelatin in situ forming hydrogel for enhanced bone regeneration. J Biomed Mater Res Part A 2018;106:531–542.

[16] Santos SG, Lamghari M, Almeida CR, Oliveira MI, Neves N, Ribeiro AC, Barbosa JN, Barros R, Maciel J, Martins MCL, Gonçalves RM, Barbosa MA. Adsorbed fibrinogen leads to improved bone regeneration and correlates with differences in the systemic immune response. Acta Biomater 2013;9:7209–7217.

[17] Vyas V, Kaur T, Thirugnanam A. Chitosan composite three dimensional macrospheric scaffolds for bone tissue engineering. Int J Biol Macromol 2017;104 (Pt B):1946–1954.

[18] Mantripragada VP, Jayasuriya AC. Bone regeneration using injectable BMP-7 loaded chitosan microparticles in rat femoral defect. Mater Sci Eng C Mater Biol Appl 2016;63:596–608.

[19] Vasconcelos DP, Costa M, Amaral IF, Barbosa MA, Águas AP, Barbosa JN. Development of an immunomodulatory biomaterial: Using resolvin D1 to modulate inflammation. Biomaterials 2015; 53:566–573.

[20] Vasconcelos DP, Fonseca AC, Costa M, Amaral IF, Barbosa MA, Águas AP, Barbosa JN. Macrophage polarization following chito- san implantation. Biomaterials 2013;34:9952–9959.

[21] Le Guehennec L, Goyenvalle E, Aguado E, Houchmand-Cuny M, Enkel B, Pilet P, Daculsi G, Layrolle P. Small-animal models for testing macroporous ceramic bone substitutes. J Biomed Mater Res B Appl Biomater 2005;72:69–78.

[22] Montes GS, Junqueira LC. The use of the Picrosirius-polarization method for the study of the biopathology of collagen. Mem Inst Oswaldo Cruz 1991;86 Suppl 3:1–11.

[23] Vieira AE, Repeke CE, Ferreira Junior S. d B, Colavite PM, Biguetti CC, Oliveira RC, Assis GF, Taga R, Trombone APF, Garlet GP. Intramembranous bone healing

process subsequent to tooth extraction in mice: micro-computed tomography, histomorpho- metric and molecular characterization. PloS One 2015;10: e0128021.
[24] Shadjou N, Hasanzadeh M. Graphene and its nanostructure derivatives for use in bone tissue engineering: Recent advances. J Biomed Mater Res Part A 2016;104:1250–1275.
[25] Celikkin N, Mastrogiacomo S, Jaroszewicz J, Walboomers XF, Swieszkowski W. Gelatin methacrylate scaffold for bone tissue engineering: The influence of polymer concentration. J Biomed Mater Res Part A 2018;106:201–209.
[26] Masalskas BF, Martins Júnior W, Leoni GB, Faloni A. P d S, Marcaccini AM, Silva Sousa YTC, Castro-Raucci L. M S d. Local delivery of strontium ranelate promotes regeneration of critical size bone defects filled with collagen sponge. J Biomed Mater Res Part A 2018;106:333–341.
[27] Brown BN, Londono R, Tottey S, Zhang L, Kukla KA, Wolf MT, Daly KA, Reing JE, Badylak SF. Macrophage phenotype as a predictor of constructive remodeling following the implantation of biologically derived surgical mesh materials. Acta Biomater 2012; 8:978–987.
[28] Hubbell JA, Thomas SN, Swartz MA. Materials engineering for immunomodulation. Nature 2009;462:449–460.
[29] Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. Nature 2013;496:445– 455.
[30] Vasconcelos DM, Gonçalves RM, Almeida CR, Pereira IO, Oliveira MI, Neves N, Silva AM, Ribeiro AC, Cunha C, Almeida AR, Ribeiro CC, Gil AM, Seebach E, Kynast KL, Richter W, Lamghari M, Santos SG, Barbosa MA. Fibrinogen scaffolds with immunomodulatory properties promote in vivo bone regeneration. Biomaterials 2016;111:163–178.
[31] Viguet-Carrin S, Garnero P, Delmas PD. The role of collagen in bone strength. Osteoporos Int 2006;17:319–336.
[32] Gallo J, Goodman SB, Konttinen YT, Raska M. Particle disease: biologic mechanisms of periprosthetic osteolysis in total hip arthroplasty. Innate Immun 2013;19:213–224.
[33] Wang X, Bank RA, TeKoppele JM, Agrawal CM. The role of colla- gen in determining bone mechanical properties. J Orthop Res 2001;19:1021.
[34] Junqueira LC, Roscoe JT. Reduced collagen content and fibre bundle disorganization in skin biopsies of patients with Ehlers- Danlos syndrome.

Histochem J 1985;17:1197–1202.

[35] Junqueira LC, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochem J 1979;11:447–455.

[36] Cavallo JA, Roma AA, Jasielec MS, Ousley J, Creamer J, Pichert MD, Baalman S, Frisella MM, Matthews BD, Deeken CR. Remodeling characteristics and collagen distribution in synthetic mesh materials explanted from human subjects after abdominal wall reconstruction: an analysis of remodeling characteristics by patient risk factors and surgical site classifications. Surg Endosc 2014;28:1852–1865.

[37] Peeters E, De Hertogh G, Junge K, Klinge U, Miserez M. Skin as marker for collagen type I/III ratio in abdominal wall fascia. Hernia 2014;18:519–525.

[38] Spite M, Claria J, Serhan CN. Resolvins, specialized proresolving lipid mediators, and their potential roles in metabolic diseases. Cell Metab 2014;19:21–36.

[39] Titos E, Rius B, González-Périz A, Lopez-Vicario C, Morán-Salvador E, Martínez-ClementeM,ArroyoV,ClàriaJ.ResolvinD1 and its precursor docosahexaenoic acid promote resolution of adipose tissue inflammation by eliciting macrophage polarization toward an M2-like phenotype. J Immunol 2011;187:5408–5418.

[40] Benabdoune H, Rondon E-P, Shi Q, Fernandes J, Ranger P, Fahmi H, Benderdour M. The role of resolvin D1 in the regulation of inflammatory and catabolic mediators in osteoarthritis. Inflamm Res 2016;65:635–645.

[41] Kain V, Ingle KA, Colas RA, Dalli J, Prabhu SD, Serhan CN, Joshi M, Halade GV. Resolvin D1 activates the inflammation resolving response at splenic and ventricular site following myocardial infarction leading to improved ventricular function. J Mol Cell Cardiol 2015;84:24–35.

[42] Hsiao H-M, Sapinoro RE, Thatcher TH, Croasdell A, Levy EP, Fulton RA, Olsen KC, Pollock SJ, Serhan CN, Phipps RP, Sime PJ. A novel anti-inflammatory and pro-resolving role for resolvin D1 in acute cigarette smoke-induced lung inflammation. PloS One 2013;8:e58258.

[43] Claes L, Recknagel S, Ignatius A. Fracture healing under healthy and inflammatory conditions. Nat Rev Rheumatol 2012;8:133–143.

# CHAPTER VI

## ORIGINAL RESEARCH WORK

### DEVELOPMENT OF A STRATEGY TO INCREASE THE RELEASE OF RESOLVIN D1 THROUGH THE TIME

### ARTICLE IV

"Solid lipid nanostructures as a novel delivery system for Resolvin D1: improvement of Resolvin D1 half-life. ***In preparation.***

**Solid lipid nanostructures as a novel delivery system for Resolvin D1: improvement of Resolvin D1 half life**

Daniela P. Vasconcelos[1,2,3], Mário A. Barbosa[1,2,3], Salette Reis[4,] Cláudia Pinho[4] & Judite N. Barbosa[1,2,3]

[1]i3S-instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal

[2]INEB, Instituto de Engenharia Biomédica, Universidade do Porto, Porto, Portugal

[3]ICBAS - Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Porto, Portugal

[4]LAQV, REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal

**ABSTRACT**

The aim of the present work was to develop a strategy for the sustained release throughout time and also to improve the stability of Resolvin D1 (RvD1), using solid lipid nanoparticles (SLNs). RvD1-loaded SLNs were successfully developed by hot ultrasonication method. All the developed formulations were systematically characterized regarding their diameter, polydispersity index (PDI) and zeta potential (ZP). The results showed a significant decrease of SLNs size after RvD1 incorporation comparing with unloaded-SLNs, revealing that an interaction between RvD1 and the lipid nanoparticles occurs. Cytotoxic analysis revealed that RvD1-loaded SLNs and unloaded-SLNs are non-toxic for human macrophages. The biological activity of RvD1-loaded SLNs one week after preparation indicates that the phospholipid layer has a protective effect in RvD1, suggesting that the biological half-life of this pro-resolving mediator can be increased by the association with these lipidic nanostructures.

***KEYWORDS***

Resolvin D1, solid lipid nanoparticles, immunomodulation, macrophages

## 1. INTRODUCTION

The process of implantation of a biomaterial causes injury to tissue and organs of the host leading to the immediate onset of an acute inflammatory response. Acute inflammatory response is mediated by a plethora of well-known chemical messengers, including cytokines, chemokines and lipid-derived mediators released by immune cells [1-3]. The perpetuation of the inflammatory cascade culminates either in resolution of inflammation, return to homeostasis and tissue healing or in chronic inflammation and biomaterial impairment.

The resolution of inflammation is an active process controlled by endogenous mediators, with selective actions on inflammatory cells. The resolution phase of the inflammatory response is characterized by the local production of anti-inflammatory and specialized proresolving mediators (SPMs) such as lipoxins, protectins, maresins and resolvins [4]. These mediators exert their anti-inflammatory roles, acting as stop signals of inflammation, by blocking the further recruitment of polymorphonuclear cells (PMNs), stimulating nonphlogistic recruitment of monocytes and also the uptake of apoptotic PMNs by macrophages [5]. SPMs are derived from different polyunsatured fatty acids (omega-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) [3].

Several studies report the biological actions of SPMs, namely, their role in metabolic diseases in the resolution of acute inflammation and tissue repair. It has been described that SPMs are beneficial for tissue repair in different models of injury, including bone [6]. Kruger MC *et al*. showed that SPMs inhibit inflammation that induces bone resorption and therefore provide a mechanism by which the SPMs have a protective role against bone loss [7]. In the adipose tissue, RvD1 enhances wound healing by promoting macrophage polarization towards the anti-inflammatory M2 phenotype [8, 9]. Resolvin D1 accelerates the resolution of lung inflammation after smoke exposure [10] and has been shown to have a therapeutic effect controlling acute respiratory syndrome [11]. Benabdoune H. and colleagues showed that RvD1 suppressed several factors that are involved in osteoarthritis, inhibiting IL-1$\beta$ induced activation of NF-$\kappa$B/p65, p38MAPK and JNK (1/2) [12]. So, pro-resolving mediators are promising endogenous natural mediators that could be used for several applications.

Nowadays, the usage of biomaterials for the improvement of tissue healing has being pointed as the future for tissue repair, being the materials with immunomodulatory properties the most promising in the tissue engineering field [13]. One of the strategies that can be used to improve tissue repair, through the modulation of immune response is the delivery of anti-inflammatory mediators, namely, the delivery of specific SPMs. The main purpose of delivering SMPs is the modulation of macrophage phenotype, from a pro-

inflammatory M1 phenotype to a pro-resolving, anti-inflammatory M2 phenotype [14]. In a previous work, we showed, in an *in vivo* model of inflammation (air pouch model), that successive local administrations of RvD1, modulate the inflammatory response to biomaterials driving macrophage polarization to an anti-inflammatory pro-reparative phenotype (M2 phenotype) and significantly decreased the thickness of the fibrous capsule around the implant [15]. Taking these results in account we decided to use 3D Ch scaffolds as a delivery system for RvD1. Using the same *in vivo* model and after 4 days of implantation we observed the same tendency, the developed material was able to modulate the macrophage response to implanted materials [16]. Considering the previous results, we verified in an *in vivo* model of tissue repair, if the developed material was able to improve bone tissue healing. The results showed that the material developed was able to increase the trabecular thickness and Coll type I in the critical size bone defect model. However, we observed only a slightly increase in the new bone formation without statistical significance [17]. These results could be related with the fact that RvD1, is rapidly inactivated by 15-prostaglandin dehydrogenase/eicosanoid oxidoreductase (EOR). RvD1 is converted by EOR, 25 min after incubation with the enzyme [18]. So, the time and the amount of RvD1 released might not be enough to induce a higher improvement in tissue repair.
Solid lipid nanoparticles, have shown to be one of the most promising delivery systems of hydrophobic drugs [19]. Solid lipid nanoparticles comprise a physiological compatible solid lipid core and an amphiphilic surfactant shell. These particles can improve the solubility of poorly soluble drugs through their incorporation in the lipid matrix and are able to improve physical stability, protection from *in vivo* degradation and controlled release [20]. Other advantages include low production cost and easy scale up.

Thus, the main purpose of this work was to develop a new approach able to obtain a sustained release of RvD1 through time and improve its half-life activity. In order to achieve this objective, SLNs were used as delivery systems. Production and characterization methodologies were set up and primary human macrophages were used to accessed SLNs cytotoxicity and RvD1 biological activity.

## 2. MATERIALS & METHODS

### *2.1. SLNs preparation*

SLNs were produced by hot ultrasonication [21] using 250 mg of Precirol ATO5® (Gattefosé, France) and 30 mg of Tween 80® (Merck, Germany). Briefly, the lipid (Precirol ATO5®) and Tween80® were weighted and heated together at 65°C to promote their mixture. Ultrapure water (5 mL) preheated at 65°C was added. Afterward, a sonicator was used (Vibra-Cell model VCX 130 equipped with a VC 18 probe, Sonics and Materials Inc., Newtown, USA), with a tip diameter of 1/4" (6 mm), at 70% amplitude for 5 min to obtain the SLNs. For the production of RvD1-loaded SLNs, RvD1 (Cayman Chemical Company, USA) was added (1 μM) before the addition of hot ultrapure water.

### *2.2. SLNs characterization*

The size and surface charge (ξ-potential) of the produced SLNs were characterized by dynamic light scattering (DLS) and electrophoretic light scattering (ELS) respectively, using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). Diluted NLC (1:50 in ultrapure water) were placed on a disposable capillary cell and triplicate measurements were conducted at a backscattering angle of 173º at 25°C.

The SLNs morphology was evaluated by NanoSight®, using a NanoSight NS300 (Malvern Instruments, UK). For NanoSight® analysis, NLC were diluted (1:40000) with ultrapure water and morphology was evaluated.

### *2.3. Human macrophages*

Human monocytes were isolated by negative selection from buffy coats of healthy donors by using RosetteSep human monocyte enrichment cocktail (StemCell Technologies), as previously described [22, 23]. The buffy coats were provided by the Immunohemotherapy Department of Centro Hospitalar São João (CHSJ) from Porto, Portugal with the approval of the clinical ethical committee (Protocol reference 260/11). Cells were resuspended in complete medium (RPMI 1640 medium (Corning) supplemented with 10% FBS (Biowest), 100 U/mL penicillin, 100 μg/mL streptomycin (both from Immunotools). For macrophage differentiation, cells were plated in TCPS at a confluence of $0.5 \times 10^6$ monocytes/well. Monocytes were cultured for 7 days in complete medium in the absence of M-CSF or others exogenous factors.

### *2.4. Cell metabolic activity*

In order to assess the biocompatibility of SLNs, cytotoxicity studies were performed using human macrophages. RvD1 – Loaded SLNs (60 nM – 30 μL), Unloaded-SLNs (30 μL) or RvD1 free solution (60 nM) were added to macrophages for 1h or 3h, and cells without treatment were used as controls. The metabolic activity of these cells was determined by resazurin reduction assay. Briefly, 1h or 3h after SLNs addition, cells were incubated with resazurin redox dye (0.01 mg/mL) (Sigma-Aldrich) for 4h at 37 °C and 5% $CO_2$. Fluorescence intensity was measured at 590 nm in a synergy Mx plate reader (BioTek), and values normalized to unstimulated cells.

### *2.5. Cytokine Profile*

The cytokine profile after macrophage incubation with SLNs was assessed by ELISA assays. Macrophages were pre-treated with SLNs for 1 h, washed and stimulated with LPS for 2 h (10 ng/mL). The supernatants were recovery and the levels of IL-6, TNF-α, IL-1β, CCL2 and IL-10 were measured by LegendMax Elisa (Biolegend), according to the supplier indications.

### *2.6. Statistical analysis*

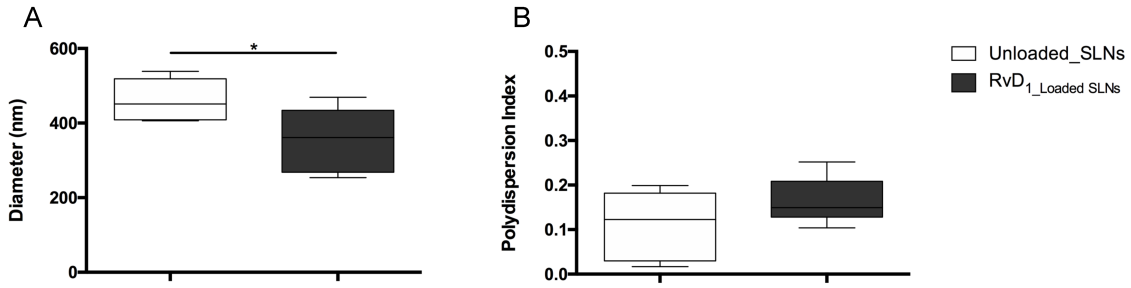
Comparisons of multiple groups were analysed by one-way analysis of variance ANOVA with Kruskal-Wallis test with Dunn's multiple comparisons test using Prism 6 software (GraphPad). *P*-values <0.05 were considered statistically significant.

## 3. RESULTS

### *3.1. Resolvin D1 nanoencapsulation*

SLNs size and ξ-potential were determined by DLS and ELS. Unloaded-SLNs (without RvD1) presented diameters of 467 ± 60 nm. After RVD1 incorporation, SLNs diameter significantly decrease, exhibiting diameters of 351 ± 93 nm (Fig. 1A). Moreover, SLNs presented a rather homogeneous size distribution with low polydispersion index (<0.2), for RvD1-loaded SLNs ~0.17 and for Unloaded SLNs ~0,09 (Fig. 1B). Surface ξ-potential of SLNs in water was around -39 mV ± 11 mV for the Unloaded-SLNs and -37 mV ± 3 for RvD1-loaded SLNs (Fig. 1C).

The morphology of these SLNs (unloaded and RvD1-loaded) was observed by Nano Sight. Images from Fig. 2 demonstrate that SLNs displayed an almost spherical morphology and were not aggregated.




D

| Unloaded-SLNs | Size | PDI | Zeta |
|---|---|---|---|
| Average | 467 | 0.09 | -39 |
| SD | 60 | 0.07 | 11 |

| RvD1 loaded-SLNs | Size | PDI | Zeta |
|---|---|---|---|
| Average | 351 | 0.17 | -37 |
| SD | 93 | 0.04 | 3 |

**Figure 8:** SLNs characterization. SLNs were characterized in terms of: diameter (A); podydispersion index (B) and charge surface (ξ-potential) (C) performed using dynamic light scattering at 25ºC. *p<0.05; statistically significant differences between unloaded-SLNs and RvD1-loaded SLNs (Kruskal-Wallis test). Summary of all data (D) (n>3).

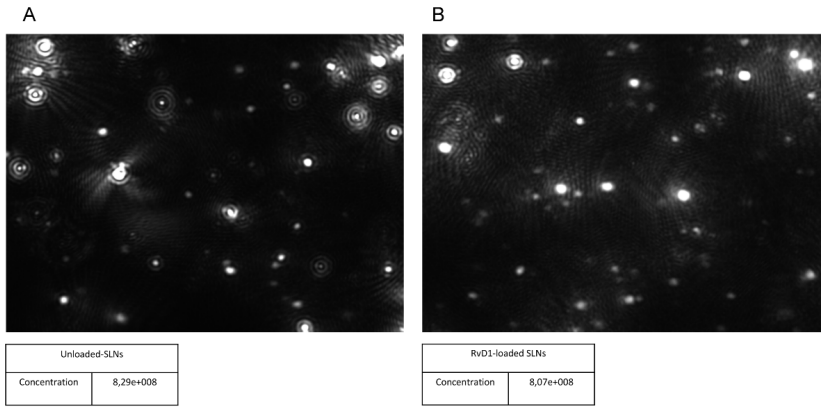



**Figure 9:** SLNs morphology characterization. Unloaded-SLNs (A) and RvD1-Loaded SLNs (B) morphology was evaluated by Nanosight®.

### *3.2. Metabolic activity of macrophages after incubation with SLNs*

RvD1-loaded SLNs development aims to modulate macrophage response. Thus, human primary macrophages were used for the cell viability analysis. Studies were conducted with unloaded, RvD1-loaded SLNs and RvD1 free solution. We decided to use a concentration of nanoparticles corresponding to 60 nM of RvD1. The concentration of RvD1 was decided taking in account what is described in the literature as having an anti-inflammatory effect [24]. Unloaded, RvD1-loaded SLNs and RvD1 free solution were incubated with human macrophages during 1h and 3h. The results (Fig. 3) showed that there was not a significant decrease in cell viability caused by the presence of SLNs (Unloaded and RvD1-loaded) compared to the controls. These results showed that the nanoparticles were safe as the viability of the cells was superior to 80%.

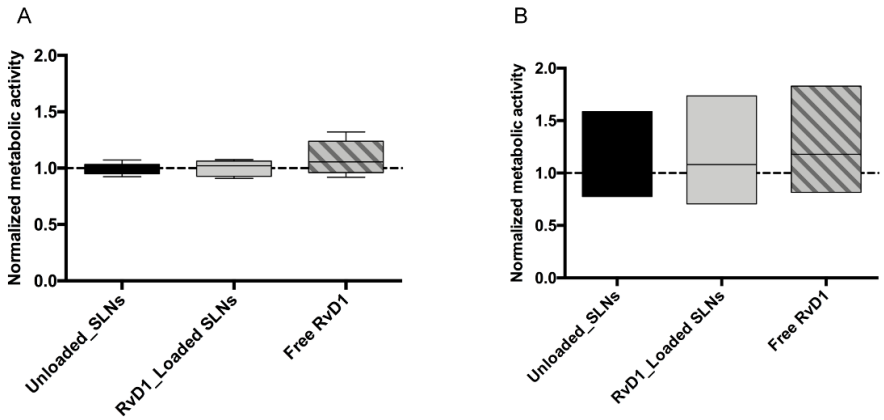



**Figure 10:** Cell viability of human macrophages upon exposure to SNLs. (A) Cell viability 1 hr after incubation with SLNs (n=6); (B) cell viability 3hr after SLNs incubation (n=3).

### *3.3. Anti-inflammatory properties of SLNs*

To assess if RvD1-loaded SLNs were able to modulate the macrophage response to LPS stimulus, cytokine profile of macrophages was analysed. Interestingly, our results showed that macrophages treated with RvD1-Loaded SLNs (even several days after its preparation) were able to reduce the production of IL-6 and TNF-α to values similar to the macrophages that were pre-treated with RvD1 free solution (fresh solution) (Fig. 4A, B). So, these results showed that SLNs were able to increase the half-life of RvD1. No differences were found in CCL2 and IL-10 production, indicating that RvD1 only affects the NF-κB pathway (Fig. 4C, D).

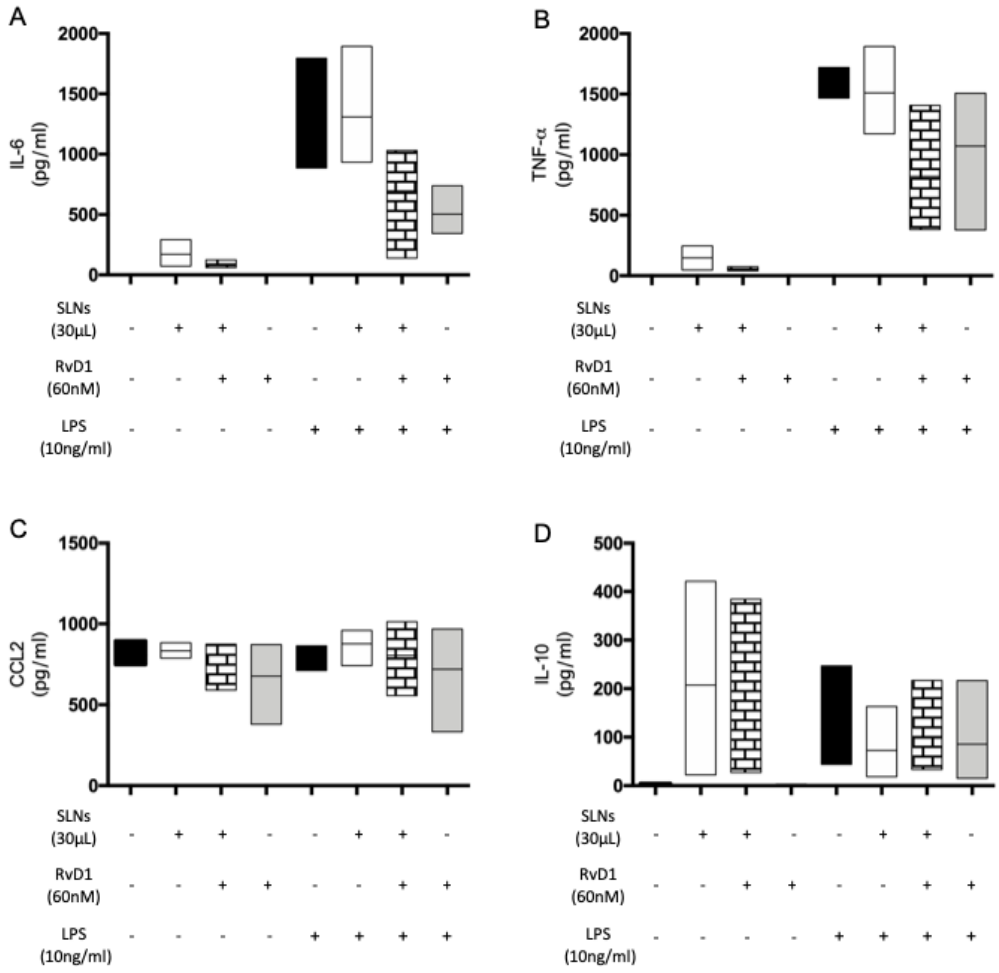



**Figure 11:** Cytokine profile after SLNs pre-treatment. After 7 days of culture, macrophages were pre-treated with SLNs (Unloaded or RvD1-loaded) and RvD1 free solution for 1 hr, after that, cells were washed and stimulated with LPS (10ng/ml) for 2hr. The supernatants were analysed for IL-6 (A); TNF-α (B); CCL2 (C) and IL-10 (D) by ELISA (n=3).

## 4. DISCUSSION

The intensity of the inflammatory response to tissue injury dictates the fate of tissue repair. An immune response well balanced will lead to an efficient tissue healing. However, when the inflammatory response to tissue injury is not well controlled can become chronic leading to fibrosis and the impairment of tissue function [25]. The usage of biomaterials to improve tissue repair has been pointed as a good strategy, since these materials can work as template for new tissue formation and can also be modified in order to give rise to a desired immune response, improving new tissue formation.

So, the development of immunomodulatory materials is the new trend in the tissue engineering field [13]. Different strategies can be used to obtain a desire immune response. Physical and chemical properties of materials can be changed in order to obtain different immune behaviours. The material itself can be used as a delivery platform for immune modulators (pro- or anti-inflammatory mediators). Recently, was suggested a more complex strategy, the “sequential immunomodulation” that comprise the stimulation of an initial pro-inflammatory response after material implantation followed by the initiation of the resolution phase of the immune response [14].

The demonstration of the key role of macrophages in tissue repair, prompts the search for new strategies to modulate the response of these cells to biomaterials, and consequently influencing the functional outcome of the implanted material. In an initial phase M1 macrophages are important in fighting pathogens through the secretion of pro-inflammatory mediators and later M2 macrophages have an important role in the wound healing, through secretion of anti-inflammatory mediators and promotion of extracellular matrix (ECM) remodelling [26].

Specialized pro resolving mediators are endogenous mediators that are actively involved in the resolution of the inflammatory response, providing a huge potential for novel therapeutic approaches [27]. We have recently reported that two of these mediators, lipoxin A4 (LxA4) and RvD1 were able to modulate the macrophage response to implanted 3D Ch scaffolds with 15% degree of acetylation (DA) [15]. We have demonstrated that Ch scaffolds with 15% DA induce the increase of M1 macrophages in the implant site and also the amount of pro-inflammatory cytokines [28]. Successive administrations of LxA4 and RvD1 change the polarization phenotype of macrophages from a pro-inflammatory M1 phenotype to an anti-inflammatory, pro-resolution M2 phenotype [15]. Thus, we were able to induce a sequential immunomodulation. To translate this knowledge into a more effective therapy *in vivo*, we

developed a strategy to incorporate the RvD1 into 3D Ch scaffolds. Chitosan scaffolds work as delivery system for RvD1 [16]. Since the developed material was capable to modify the macrophage response in an *in vivo* model of inflammation, we decided to test it *in vivo* in a model of tissue repair. For that a critical size bone defect was used. Two months after implantation we observed a slightly increase in the new bone formation, a significant increase in trabecular thickness and Coll type I [17]. The results were not as expected, since we were predicting larger amount of new bone formation. In fact, this could be related with the tissue injury size that needs several days to be resolved. *In vitro* studies showed that RvD1 incorporated in the scaffolds is totally released in 72h [16] and therefore, we hypothesize that the amount of RvD1 is not enough to induce the expected modulatory effect.

Taking this in account we develop a new strategy to increase the release time of RvD1 together with the improvement of RvD1 half-life. RvD1 has a half-life of minutes [18], and it is crucial that the delivery system is able to improve its stability. It has been described that phospholipids increase SPMs stability. Studies were performed using liposomes, which are extremely expensive [29, 30]. Meanwhile, Solid lipid nanoparticles (SLNs) are pointed as a noble delivery system of hydrophobic drugs (e.g. lipids) [19]. The costs of SLNs production are low and in addition are easily scale up [31]. Thus, we decided to use SLNs as the delivery system of RvD1. In this study, SLNs were synthesized using hot ultrasonication, avoiding the use of organic solvents. In addition, they present low cytotoxicity due to their lipid biodegradable and biocompatible composition recognized as safe by FDA. RvD1-loaded SLNs with ~350nm of diameter, and negative charge (around – 37mV) were successfully produced. These RvD1-loaded SLNs were not cytotoxic to human macrophages, since a decrease in cell metabolism was not observed.

Aiming to develop a new strategy to increase the half-life of RvD1 one of our main interests was to investigate the functional consequences of the pre-treatment induced by SLNs (unloaded and RvD1-loaded) prepared days before usage, in human macrophages stimulated with lipopolysaccharide (LPS). Here we demonstrated that RvD1-loaded SLNs were able to decrease the levels of the pro-inflammatory cytokines, IL- 6 and TNF-$\alpha$ close to values of cells that were treated with fresh RvD1 free solution. These results are in accordance with the literature since RvD1 inhibited the phosphorylation of TAK1, a key upstream regulatory kinase common to both MAP kinase and NF-$\kappa$B pathways, decreasing IL-6 and TNF-$\alpha$ [32, 33]. No differences were observed with other cytokines, namely CCL2, IL-10 and IL-1$\beta$ (data not shown).

Further studies concerning the analysis of the release profile of RvD1 would be of interest to validate the developed strategy.

## 5. CONCLUSIONS

Based on the results obtained until now, it can be concluded that we were able to optimize RvD1 stability and SLNs are a promising platform to deliver RvD1, since RvD1-loaded SLNs showed an anti-inflammatory effect, while being non-toxic to primary human macrophages. The results obtained until now are promising and further studies will be performed, namely the entrapment efficiency and release studies.

## *ACKNOWLEDGMENTS*

We thank Catarina Sebra for the material support for the SLNs experiments and Andreia Silva for the help with Nanosight® equipment. This work was financed by: FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through FCT - Fundação para a Ciência e a Tecno- logia in the framework of the project “Institute for Research and Innovation in Health Sciences” (POCI-01-0145-FEDER-007274) and NORTE-01- 0145-FEDER-000012, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). Daniela P. Vasconcelos is funded by FCT PhD Programmes and by Programa Operacional Potencial Humano (POCH), BiotechHealth Programme (Doctoral Programme on Cellular and Molecular Biotechnology Applied to Health Sciences - reference PD/00016/2012), and through the PhD studentship PD/BD/114011/2015.

**REFERENCES**

[1] C. Nathan, Points of control in inflammation, Nature 420(6917) (2002) 846-52.
[2] J.W. Griffith, C.L. Sokol, A.D. Luster, Chemokines and chemokine receptors: positioning cells for host defense and immunity, Annu Rev Immunol 32 (2014) 659-702.
[3] C.N. Serhan, J. Savill, Resolution of inflammation: the beginning programs the end, Nature immunology 6(12) (2005) 1191-7.
[4] M.O. Freire, T.E. Van Dyke, Natural resolution of inflammation, Periodontology 2000 63(1) (2013) 149-64.
[5] C.D. Buckley, D.W. Gilroy, C.N. Serhan, Proresolving lipid mediators and mechanisms in the resolution of acute inflammation, Immunity 40(3) (2014) 315-27.
[6] L. Gao, D. Faibish, G. Fredman, B.S. Herrera, N. Chiang, C.N. Serhan, T.E. Van Dyke, R. Gyurko, Resolvin E1 and chemokine-like receptor 1 mediate bone preservation, Journal of immunology 190(2) (2013) 689-94.
[7] M.C. Kruger, M. Coetzee, M. Haag, H. Weiler, Long-chain polyunsaturated fatty acids: selected mechanisms of action on bone, Progress in lipid research 49(4) (2010) 438-49.
[8] M. Spite, J. Claria, C.N. Serhan, Resolvins, specialized proresolving lipid mediators, and their potential roles in metabolic diseases, Cell metabolism 19(1) (2014) 21-36.
[9] Y. Tang, M.J. Zhang, J. Hellmann, M. Kosuri, A. Bhatnagar, M. Spite, Proresolution therapy for the treatment of delayed healing of diabetic wounds, Diabetes 62(2) (2013) 618-27.
[10] H.M. Hsiao, R.E. Sapinoro, T.H. Thatcher, A. Croasdell, E.P. Levy, R.A. Fulton, K.C. Olsen, S.J. Pollock, C.N. Serhan, R.P. Phipps, P.J. Sime, A novel anti-inflammatory and pro-resolving role for resolvin D1 in acute cigarette smoke-induced lung inflammation, PloS one 8(3) (2013) e58258.
[11] Y. Gao, H. Zhang, L. Luo, J. Lin, D. Li, S. Zheng, H. Huang, S. Yan, J. Yang, Y. Hao, H. Li, F. Gao Smith, S. Jin, Resolvin D1 Improves the Resolution of Inflammation via Activating NF-kappaB p50/p50-Mediated Cyclooxygenase-2 Expression in Acute Respiratory Distress Syndrome, Journal of immunology (2017).
[12] H. Benabdoune, E.P. Rondon, Q. Shi, J. Fernandes, P. Ranger, H. Fahmi, M. Benderdour, The role of resolvin D1 in the regulation of inflammatory and catabolic mediators in osteoarthritis, Inflamm Res 65(8) (2016) 635-45.
[13] A. Vishwakarma, N.S. Bhise, M.B. Evangelista, J. Rouwkema, M.R. Dokmeci, A.M. Ghaemmaghami, N.E. Vrana, A. Khademhosseini, Engineering Immunomodulatory Biomaterials To Tune the Inflammatory Response, Trends Biotechnol 34(6) (2016) 470-82.
[14] Z. Julier, A.J. Park, P.S. Briquez, M.M. Martino, Promoting tissue regeneration by modulating the immune system, Acta biomaterialia 53 (2017) 13-28.

[15] D.P. Vasconcelos, M. Costa, I.F. Amaral, M.A. Barbosa, A.P. Aguas, J.N. Barbosa, Modulation of the inflammatory response to chitosan through M2 macrophage polarization using pro-resolution mediators, Biomaterials 37 (2015) 116-23.

[16] D.P. Vasconcelos, M. Costa, I.F. Amaral, M.A. Barbosa, A.P. Aguas, J.N. Barbosa, Development of an immunomodulatory biomaterial: Using resolvin D1 to modulate inflammation, Biomaterials 53 (2015) 566-73.

[17] D.P. Vasconcelos, M. Costa, N. Neves, J.H. Teixeira, D.M. Vasconcelos, S.G. Santos, A.P. Aguas, M.A. Barbosa, J.N. Barbosa, Chitosan porous 3D scaffolds embedded with resolvin D1 to improve in vivo bone healing, Journal of biomedical materials research. Part A 106(6) (2018) 1626-1633.

[18] Y.P. Sun, S.F. Oh, J. Uddin, R. Yang, K. Gotlinger, E. Campbell, S.P. Colgan, N.A. Petasis, C.N. Serhan, Resolvin D1 and its aspirin-triggered 17R epimer. Stereochemical assignments, anti-inflammatory properties, and enzymatic inactivation, The Journal of biological chemistry 282(13) (2007) 9323-34.

[19] M. Narvekar, H.Y. Xue, J.Y. Eoh, H.L. Wong, Nanocarrier for poorly water-soluble anticancer drugs--barriers of translation and solutions, AAPS PharmSciTech 15(4) (2014) 822-33.

[20] A.A. Attama, C.E. Umeyor, The use of solid lipid nanoparticles for sustained drug release, Ther Deliv 6(6) (2015) 669-84.

[21] A.C. Vieira, L.L. Chaves, M. Pinheiro, D. Ferreira, B. Sarmento, S. Reis, Design and statistical modeling of mannose-decorated dapsone-containing nanoparticles as a strategy of targeting intestinal M-cells, International journal of nanomedicine 11 (2016) 2601-17.

[22] A.P. Cardoso, M.L. Pinto, A.T. Pinto, M.I. Oliveira, M.T. Pinto, R. Goncalves, J.B. Relvas, C. Figueiredo, R. Seruca, A. Mantovani, M. Mareel, M.A. Barbosa, M.J. Oliveira, Macrophages stimulate gastric and colorectal cancer invasion through EGFR Y(1086), c-Src, Erk1/2 and Akt phosphorylation and smallGTPase activity, Oncogene 33(16) (2014) 2123-33.

[23] F. Castro, M.L. Pinto, A.M. Silva, C.L. Pereira, G.Q. Teixeira, M. Gomez-Lazaro, S.G. Santos, M.A. Barbosa, R.M. Goncalves, M.J. Oliveira, Pro-inflammatory chitosan/poly(gamma-glutamic acid) nanoparticles modulate human antigen-presenting cells phenotype and revert their pro-invasive capacity, Acta biomaterialia 63 (2017) 96-109.

[24] G. Li, Z. Chen, O.M. Bhat, Q. Zhang, J.M. Abais-Battad, S.M. Conley, J.K. Ritter, P.L. Li, NLRP3 inflammasome as a novel target for docosahexaenoic acid metabolites to abrogate glomerular injury, J Lipid Res 58(6) (2017) 1080-1090.

[25] S.A. Eming, T. Krieg, J.M. Davidson, Inflammation in wound repair: molecular and cellular mechanisms, The Journal of investigative dermatology 127(3) (2007) 514-25.

[26] T.A. Wynn, K.M. Vannella, Macrophages in Tissue Repair, Regeneration, and Fibrosis, Immunity 44(3) (2016) 450-462.

[27] C.N. Serhan, Pro-resolving lipid mediators are leads for resolution physiology, Nature 510(7503) (2014) 92-101.

[28] D.P. Vasconcelos, A.C. Fonseca, M. Costa, I.F. Amaral, M.A. Barbosa, A.P. Aguas, J.N. Barbosa, Macrophage polarization following chitosan implantation, Biomaterials 34(38) (2013) 9952-9.

[29] S. Fiore, C.N. Serhan, Phospholipid bilayers enhance the stability of leukotriene A4 and epoxytetraenes: stabilization of eicosanoids by liposomes, Biochemical and biophysical research communications 159(2) (1989) 477-81.

[30] V. Kain, K.A. Ingle, R.A. Colas, J. Dalli, S.D. Prabhu, C.N. Serhan, M. Joshi, G.V. Halade, Resolvin D1 activates the inflammation resolving response at splenic and ventricular site following myocardial infarction leading to improved ventricular function, Journal of molecular and cellular cardiology 84 (2015) 24-35.

[31] L.L. Chaves, S. Lima, A.C.C. Vieira, D. Ferreira, B. Sarmento, S. Reis, Overcoming clofazimine intrinsic toxicity: statistical modelling and characterization of solid lipid nanoparticles, Journal of the Royal Society, Interface / the Royal Society 15(139) (2018).

[32] H.M. Hsiao, T.H. Thatcher, E.P. Levy, R.A. Fulton, K.M. Owens, R.P. Phipps, P.J. Sime, Resolvin D1 attenuates polyinosinic-polycytidylic acid-induced inflammatory signaling in human airway epithelial cells via TAK1, Journal of immunology 193(10) (2014) 4980-7.

[33] Z. Liao, J. Dong, W. Wu, T. Yang, T. Wang, L. Guo, L. Chen, D. Xu, F. Wen, Resolvin D1 attenuates inflammation in lipopolysaccharide-induced acute lung injury through a process involving the PPARgamma/NF-kappaB pathway, Respir Res 13 (2012) 110.

# CHAPTER VII

## GENERAL DISCUSSION & FUTURE PERSPECTIVES

## 1. GENERAL DISCUSSION

The work of this thesis was developed in view of enhancing the knowledge concerning macrophage response to macroscopic 3D Ch scaffolds, as well as the development of a new approach to modulate macrophage response to this scaffold leading to an improvement in tissue repair.

Tissue repair is a critical biological process, essential to the survival of all living organisms. The immune response to tissue injury is key in defining the outcome of the healing process, including the degree of damaging and the restoration of tissue integrity and function [1, 2].

Delayed bone healing or non-union fractures in response to traumatic injury, surgery or disease, has significant effect on patient´s quality of life and are a challenge in orthopedic practice. As a consequence of delayed fracture healing, bone-related medical treatments and costs increase [3]. The current “gold standard” treatment for delayed or non-union fractures is bone grafting, using an autograft or an allograft. This treatment has several drawbacks, namely the donor site morbidity and pain, the size of bone that could be harvested and the risk of disease [4, 5]. So, a more efficient and long-term treatment strategy is required. Bone grafts substitutes have been developed to facilitate the repair of natural tissues, namely 3D scaffolds have received wide attention in the past years due to their unique physical properties with controlled porosity to support bone tissue growth [6, 7]. For a correct bone repair, scaffolds need to imitate natural bone tissue, support bone growth and should elicit minimal host tissue inflammation [6, 7]. One of the most promising strategies to improve tissue repair is the use of biomaterials that allow the modulation of the inflammatory response [8, 9].

It is important to take in consideration that the process of biomaterial implantation results in damage to tissues or organs of the host. A series of events are initiated upon material implantation, starting with an acute inflammatory response that, when is not well resolved, leads to a chronic response and the deposition of a collagenous fibrous capsule around the implant, harming material function [10, 11]. So, it is of extreme relevance to understand how a specific material is recognized by immune cells.

In this doctorate, we decided to use Ch, a well know biomaterial with several remarkable properties that offer unique opportunities to the development of biomedical applications [12]. The interaction between Ch-based products and immune cells has been deeply studied over the last years by our team and others. The results obtained showed that

different chemical (DA and MW) and physical (2D films, nanoparticles, 3D structures) properties of Ch prompts different immune responses. Oliveira *et al*. verified that 2D Ch films (DA of 11%) drives anti-inflammatory macrophage polarization [13] and also showed that Ch was not able to activate the MAPK pathways ERK1/2 and JNK, TLR4 downstream pathways [14]. Our initial work demonstrated that 3D Ch scaffolds with different DA stimulates different macrophage polarization phenotypes. Macrophage polarize for a M1 pro-inflammatory phenotype in presence of 3D Ch scaffolds with higher DA (15%). On the other hand, implantation of 3D Ch scaffolds with lower DA (4%) leads to a M2 macrophage polarization [15]. Castro F et al. disclosed that Ch/poly ($\gamma$-glutamic acid) (DA of 11%) nanoparticles promote M2-to-M1 transition of macrophage polarization [16]. Tu J. *et al*. showed that in LPS-primed Caco-2 cells Ch nanoparticles were able to reduce inflammatory reaction decreasing cytoplasmic I$\kappa$B-$\alpha$ degradation and nuclear NF-$\kappa$B p65 levels [17]. Fong *et al.* suggested that biodegradable Ch microparticles induce anabolic responses in M0 (resting macrophages) and M2 but not M1 U937 macrophages, increasing IL-10 release and the release of excess IL-1r$\alpha$ over IL-1$\beta$ [18].

Although immune-modulating properties of Ch have been studied for many years, intracellular Ch response pathways have only recently start to be clarified. The activation of NLRP3 inflammasome pathway by lysosomal rupture after Ch uptake, was one of the mechanisms described has been associated to immune response to Ch-based products [19-21]. Fong *et al*. postulated that the inflammatory response to Ch depend on prior immune cell activation state and Ch dose [22]. Considering that all the studies showing Ch a potent activator of NLRP3 inflammasome were performed with nanoscale Ch-based products, and since for tissue repair macroscopic materials are required, up to date, a question was still left open: what is the role of NLRP3 inflammasome in the macrophage response to macroscopic biomaterials?

In this thesis, as the first main goal we attempted to answer this question by study the role of 3D Ch scafolds with different DA in NLRP3 inflammasome priming and activation. We start to assess the NLRP3 inflammasome in BMDM culture within 3D Ch scaffolds. Mouse bone marrow derived marocphages were plated in Ch scaffolds at day 6 of differentiation and primed at day 7 with LPS and subsequent NLRP3 activation was achieved after incubation with nigericin. One of the drawbacks for the establishment of BMDM culture into 3D Ch scaffolds was the selection for optimal time point for cell seeding. We tried different approaches such as, (i) isolation of BMDM from bone marrow and plate immediately the cells in Ch scaffolds; (ii) isolation and differentiation for 3 days in a petri dish plate, detachment and cell seeding in Ch scaffolds and finally (iii) left the BMDM differentiate for

6 days upon isolation, detach and plate BMDM in Ch scaffolds. In (i) and (ii) approaches cells were not viable, so we decided to use the (iii) methodology for the all experiments.

As described in Chapter III, in contrast to what is described in the literature with nanoscale Ch-based products, our results showed for the first time that 3D Ch scaffolds *per se* were not able to activate the inflammasome, no IL-1β release was detected with Ch scaffolds without stimulus and even after LPS priming [19-21]. More importantly, the release of IL-1β after NLRP3 inflammasome activation with nigericin was significantly decreased in BMDM seeded in 3D Ch scaffolds with lower DA (4%) when compared with cells in TCPS. A decrease in IL-1β release on cells cultured in 3D Ch with higher DA (15%) was also observed, although the difference was not statistically significant. Interestingly, we also verified that Ch scaffolds were able to modulate macrophage response to NLRP3 inflammasome activation. The maturation and release of IL-1β after inflammasome activation could lead to a type of lytic cell death known as pyroptosis [23-25]. Lactate dehydrogenase is released by dead or dying cells during pyroptosis [26], and we measured the LDH in the supernatant and found a significant decrease of LDH leakage induced by inflammasome activation in cells within Ch scaffolds with lower DA, comparing with cells in TCPS. Macrophages in Ch scaffolds with lower DA presented approximately 40% less pyroptosis than cells cultured in TCPS. Macrophages seeded on Ch scaffolds with 15% DA also present less pyroptosis than cells in TCPS, although this decrease was not statistically significant. These findings support the results obtained with IL-1β release. The inflammasome activation is an incredibly controlled process and requires two signals. The "priming" step is responsible for the transcription of NLRP3 and pro-IL-1β through the activation of NF-κB pathway. The second step signal leads to the oligomerization of NLRP3 and consequently maturation and release of IL-1β [27]. Regarding the effect of 3D Ch scaffolds on NLRP3 inflammasome priming we demonstrated that Ch scaffolds did not trigger the expression of inflammasome components, even after LPS priming. We also observed a highly decrease of pro-IL-1β expression following LPS priming in BMDM seeded on both Ch scaffolds, being the differences statistically significant for Ch scaffolds with lower DA. The analysis to the second step signal revealed that BMDM plated on Ch scaffolds with lower DA presented a significantly lower number of NLRP3 oligomers or ASC specks, comparing with cells in TCPS. This tendency was also observed for macrophages cultured in Ch scaffolds with higher DA. With these results we demonstrated that NLRP3 inflammasome activation on BMDM is strongly down-modulated in presence of 3D Ch scaffolds.

Taking in account the results with BMDM, the next step was the study of NLRP3 inflammasome activation in primary human macrophages cultured in 3D Ch scaffolds. Differently to BMDM, human macrophages were seeded in Ch scaffolds in the isolation day and left to differentiate until day 6. Consistently with what we found with BMDM, human macrophages without stimulus, were not able to induce NLRP3 inflammasome activation. In contrast to what was verified in BMDM, in human macrophages an increase of IL-1β release after LPS priming in all tested conditions was observed. It was recently described that human monocytes exhibit an alternative one-step pathway of NLRP3 activation in response to LPS alone, so it is reasonable to assume that some alternative NLRP3 activation could account for this release [28]. Importantly, after NLRP3 inflammasome activation, macrophages cultured in Ch scaffolds showed a decrease of IL-1β release, being the differences in human macrophages statistically different for cells cultured in Ch scaffolds with higher DA. Concerning the role of 3D Ch scaffolds in priming step of inflammasome activation we observed a decrease of NLRP3 expression in cells cultured in both Ch scaffolds, comparing with cells in TCPS. Concerning pro-IL-1β expression by human macrophages cultured in Ch scaffolds we did not observed the decrease verified in BMDM. Instead, we detected an increase of pro-IL-1β expression after LPS priming in cells cultured in Ch scaffolds with lower DA. It is important to notice that the increase of pro-IL-1β expression did not change after NLRP3 inflammasome activation with nigericin, demonstrating that the maturation and release of IL-1β did not occur, as showed before. As verified with BMDM, 3D Ch scaffolds also down-regulate the NLRP3 inflammasome oligomerization in human macrophages. The results of ASC specks quantification showed a decrease in the number of oligomers per cell in both Ch scaffolds. Since we observed a significant decrease of IL-1β release with Ch scaffolds, we expected to perceive the same tendency with cell dead. Surprisingly, we did not observe a significant difference in the LDH leakage among conditions. Taking in account the results with IL-1β we expected a higher percentage of LDH activity in the supernatants of cells cultured in TCPS. In the literature is generally accepted that cell rupture is correlated with IL-1β release. So, it is expected that IL-1β release is directly related with cell dead. However, recently studies presented evidences that contradict this model. Monteleone *et al.* and Evavold *et al.*, demonstrated IL-1β release in non-pyroptotic macrophages [29, 30] which can explain the results what we have obtained.

A previous work from us, demonstrated that 3D Ch scaffolds with different DA (4 and 15%) hints distinctive macrophage polarization phenotype following material implantation in an *in vivo* model of inflammation. Chitosan scaffolds with higher DA induce a typical pro-inflammatory response mediated by M1 macrophages. On the other hand, Ch scaffolds with

lower DA caused an inflammatory reaction characteristic of a macrophage M2 reparative response [15]. Following these results, we anticipated to observe clear differences in NLRP3 inflammasome activation in the studied scaffolds. These differences were not found, moreover the results on both DA were rather similar. We assume that these differences are related to the fact that *in vivo*, different cell types are present at the implant site will secrete different mediators that will have an influence in the fate of macrophage polarization in response to the implanted material.

Overall, the findings stemming from this thesis on NLRP3 inflammasome – 3D Ch-based products lead us to propose that macroscopic 3D Ch scaffolds, regardless of DA impair the NLRP3 inflammasome activation, disturbing the priming and assembly steps both in mouse and human macrophages. These outcomes provide an important understanding of the immunological properties of 3D Ch scaffolds, which will dictate a favorable fate for biomaterial integration.

Different approaches can be used to improve the healing process through immunomodulation [31]. The physicochemical properties of the material can be altered to induce different responses [15, 32], or the most common strategies reside in the delivery of pro-inflammatory modulators or anti-inflammatory/pro-resolving mediators [33]. A more complex approach is related to a sequential delivery of pro-inflammatory mediators in an early stage of the healing phase to trigger the inflammatory response and the clearance of the injured tissue, and anti-inflammatory/pro-reparative mediators in a later phase to initiate the repair/remodeling of the tissue. The sequential modulation employs a more comprehensive control throughout the tissue healing process [31]. Spiller *et al.*, revealed that the sequential delivery of IFN-γ and IL-4 to promote the M1-to-M2 transition, respectively, improve vascularization of bone scaffolds [34]. More recently Li *et al.* also showed on 5% calcium silicate/β-tricalcium phosphate scaffolds that IFN-γ release in the early stage to induce M1 phenotype, followed by release of Si prompting the switch to M2 polarization increase the formation of more blood vessels comparing with the control groups [35]. Alhamdi *et al.* described that the weakened and delayed bone healing is due to age-related alterations in bone cells and in the immune system, namely a deficient resolution of inflammation delays healing. The authors developed a strategy for temporal M1-to-M2 polarization transition and perform the proof of concept in macrophages from old mice, using biomimetic calcium phosphate coating to physically and temporally divide the pro-inflammatory stimulus IFN-γ, and the pro-reparative stimulus simvastatin. The results showed a sequential transition of M1 polarization to M2 phenotype in aged macrophages [36].

As pointed by several authors, the modulation of inflammatory response to biomaterial implantation is key for promoting tissue healing. In this work, the modulation of inflammatory response in response to implanted materials was attempted to promote tissue repair.

This led us to our second main aim that was the development of an immunomodulatory biomaterial. To achieve such purpose, we used RvD1 to modulate inflammation through M2 macrophage polarization. Resolvin D1 is an endogenous pro-resolving mediator with several functions in the resolution of inflammation. In a previous work we observed that RvD1 was able to experimentally shift the macrophage response to the implanted Ch scaffolds with 15% DA [37]. As explained before we knew for previous works that 3D Ch scaffolds with higher DA induces a typical M1 macrophage phenotype and RvD1 prompted the M1-to-M2 macrophage polarization transition. So, our main goal in this part of the work was to develop a sequential immunomodulation strategy. Resolvin D1 was added to Ch scaffolds, and the scaffolds were immediately lyophilized. It has been reported that lyophilization increase the long-term stability of pharmaceutical formulations and it is a lipid stabilizer [38, 39]. It is known that RvD1 has a half-life of minutes [40], so the effect of lyophilization after RvD1 was critical for its effective application. The release assays demonstrated that the group with lyophilized RvD1 presented a delay in the release of RvD1, but then released higher amounts of RvD1 when compared with Ch scaffolds with RvD1 without lyophilization. The total amount of RvD1 was released after 72h. The developed material was implanted in an air pouch mouse model. Interestingly, we verified with the developed material a diminishment in the total number of inflammatory cells that were recruited to the implanted site and into the 3D Ch scaffolds, together with a general decrease of pro-inflammatory cytokines like, IL-6, TNF-$\alpha$ and IL-1$\beta$. More significantly, Ch scaffolds with RvD1 lyophilized diminished the percentage of F4/80+/CCR7+ cells (M1 macrophage) and augmented the percentage of F4/80+/CD206+ cells (M2 macrophage) leading to an important increase in the M2:M1 ratio in the implanted site. A higher M2:M1 ratio has been associated with an improvement in tissue repair [41].
To our knowledge this was the first study using RvD1 incorporated into 3D Ch scaffolds as an immunomodulatory biomaterial that allowed the modulation of macrophage phenotype towards an M2 anti-inflammatory, pro-reparative phenotype.

Understanding the physiology of the bone healing process is the first step in the development of successful scaffolds for bone repair. Fracture repair is a complex highly regulated process, with consecutives phases of inflammation, repair and remodeling, that involves the interplay of many cells and their mediators [5]. Macrophages have an important

role in bone healing, they are the osteoclast precursors cells [42], are fundamental for the osteoblasts maintenance and promote intramembranous and endochondral ossification [43, 44] recruiting and stimulating MSCs differentiation [45]. Macrophages have been reported to be present throughout all bone healing phases. They are part of the acute phase response triggered by tissue injury, acting as phagocytic cells clearing cell debris and possible pathogenic invaders and secreting immune mediators that will recruit other cell types [46, 47]. Recently, Schlundt *et al.* demonstrated that a complete lack of macrophages in an early phase of bone healing, abolished the repair, impairing soft and hard callus formation leading to a complete failure in fracture repair [44]. Vi *et al.* showed that macrophages are critical for osteoblast differentiation [48]. These studies demonstrated that macrophages are important for the early as well for later phase of bone healing. Consequently, these cells represent a major target in regenerative medicine. A key element in tissue repair is the switch from the initially pro-inflammatory M1 macrophages to the anti-inflammatory, pro-reparative M2 phenotype. Disturbances at any point in the process can lead to an anomalous repair [49].

As proof of concept for the material developed in the chapter IV an *in vivo* rat femoral defect model well established in our team was used. Therefore, the main aim of this part of the work, that is detailed described in Chapter V was to verify in an *in vivo* model of bone tissue injury the ability of the developed material to improve bone healing. Each animal was implanted in the bone defect with Ch + RvD1 or Ch alone. Bone defects were analyzed two months after material implantation. We verified by micro-computed tomography a slightly increase in new bone formation (BV/TV) in the defect region in animals that received Ch + RvD1, comparing with Ch alone together with a statistically significant increase of bone trabecular thickness (Tb.Th), a reliable indicator of bone strength [50]. It is described that bone strength depends not only the quantity of bone tissue but also on its quality, being the bone quality assessed in part by the presence of Coll type I [50]. Picrosirius-polarization method with analysis of birefringent fibers was used to analyze the presence of Coll type I and Coll type III [51]. Our data demonstrated a significant increase on Coll type I fibers, as well an increase of Coll I/Coll III ratio in animals with Ch + RvD1. Higher amount of Coll type I is characterized by the organized arrangement, giving greater stiffness when compared to the disorganized nature of Coll type III fibers. So, we could conclude that RvD1 has a positive effect in bone healing, giving rise to a new bone with a higher organization and strength. Even though we have obtained interesting results, they were not as significant as expected, since we were predicting higher bone formation. The lower amount of new bone formation could be related with the fact that the tissue injury performed was very extensive and takes several days to be resolved. As the in vitro RvD1 release studies showed that

RvD1 is totally released from Ch scaffolds in 72h. We hypothesize that the amount of RvD1 released in the bone defect was not enough to induce a higher modulatory effect, or that it would be necessary to maintain the release of RvD1 after the 72h.

So, in the last chapter of this thesis (chapter VI) we decided to develop a new strategy to improve the release through the time of RvD1 together with the increase of RvD1 half-life. In order to achieve this objective, we decided to use SLNs as the delivery system for RvD1. Solid lipid nanoparticles have been described as being a rather adequate delivery system of hydrophobic drugs that are able to improve the physical stability and controlled release. Up to now we were able to optimize RvD1 stability [52, 53]. RvD1-loaded SLNs produced in the day before to be used in human macrophages showed an anti-inflammatory effect, leading to a decrease in the release of pro-inflammatory cytokines, IL-6 and TNF-$\alpha$ after LPS priming. The results obtaining until now seem to be rather promising and further developments in the framework of this thesis project would include the validation of the developed strategy with the RvD1-loaded SLNs.

Overall in the scope of this thesis, we brought new insights on the role of NLRP3 inflammasome in macrophages response to Ch, showing for the first time that macroscopic Ch-based products modulate NLRP3 inflammasome activation, impairing the priming step on primary mouse and human macrophages. We also developed, characterized and validate an immunomodulatory material that was able to promote the transition from M1 to M2 macrophage polarization in an *in vivo* model of inflammation. Finally, we verify in an *in vivo* model of bone repair, that RvD1 has an effect on bone healing, and we were able to develop a delivery system for RvD1 increasing the stability and the half-life of this pro-resolving mediator.

The work presented in this dissertation will strongly impact the community that addresses the macrophage response to biomaterials, and hopefully, also the clinical investigation to ultimately developed target materials to improve bone repair.

## 2. FUTURE PERSPECTIVES

In chapter III we describe our studies regarding NLRP3 inflammasome activation in the presence of macroscopic 3D Ch scaffolds. Following our outcomes, we believe that it will be of great interest to perform *in vivo* studies on KO mice for NLRP3 components, to explore in detail the role of NLRP3 inflammasome in the *in vivo* immune response to implanted 3D Ch scaffolds. The *in vivo* studies could further highlight the function of NLRP3 inflammasome in the immune response to implanted biomaterials.
We truly believe that understanding the signaling that is elicited by inflammasomes could be useful to abrogate fibrosis and to improve tissue healing. Therefore, modulation of inflammasome activity can be an important target to develop effective strategies for biomaterial integration which represents one of the most important challenges in the biomedical research and clinical medicine.

Afterwards we have developed an immunomodulatory biomaterial that is detailed described in chapter IV, 3D Ch scaffolds impregnated with the specialized pro-resolution mediator RvD1. The results obtained were rather interesting since we were able to control *in vivo* the macrophage phenotype. As the results were very promising, we consider that it would be of great interest to explore in detail the use of this specialized pro-resolution mediators, namely Maresin 1 (macrophage mediators in resolving inflammation) that act specifically on macrophages and have an important role in re-establishing the tissue homeostasis. We predict that the use of maresin 1 could give rise to interesting results concerning the modulation of macrophage polarization.

The results obtained on the research work described in chapter V, as explained in the previous section, were not as significant as expected which lead us to develop the strategy described in chapter VI. Nevertheless, it would be important to perform the *in vivo* studies in the rat femoral defect model using higher concentrations of RvD1 to asses if better outcomes would be achieved.In addition, it would be also interesting to perform the studies at different time points to evaluate the effect of RvD1 over time and in the different phases of bone repair and also to try to correlate the immune response observed with bone tissue repair.

Regarding the work described in the chapter VI, future studies to validate the use of SLNs as an interesting delivery system to increase the half-life and the release time of RvD1 will be crucial, namely the entrapment efficiency assay and release studies to verify if the release of RvD1 through time. Afterwards, it would be also of keen interest to verify, *in vivo*

in the model of bone tissue repair discussed in chapter V, if the RvD1-loaded SLNs have the ability to improve bone repair. In order to use RvD1-loaded SLNs at sites of bone injury, SLNs would need to be combined with Ch scaffolds, and thus allowing its implantation, making possible a controlled and localized release of RvD1 which, in our view, could improve bone healing. To achieve this purpose, we already start to explore different approaches to combine the SLNs with Ch scaffolds, but additional tests must be performed, such as, a deeply physical characterization of Ch scaffolds after the incorporation of the SLNs.

## REFERENCES

[1] S.A. Eming, P. Martin, M. Tomic-Canic, Wound repair and regeneration: mechanisms, signaling, and translation, Sci Transl Med 6(265) (2014) 265sr6.

[2] S.A. Eming, T.A. Wynn, P. Martin, Inflammation and metabolism in tissue repair and regeneration, Science 356(6342) (2017) 1026-1030.

[3] C. Schlundt, C.H. Bucher, S. Tsitsilonis, H. Schell, G.N. Duda, K. Schmidt-Bleek, Clinical and Research Approaches to Treat Non-union Fracture, Curr Osteoporos Rep 16(2) (2018) 155-168.

[4] A. Ho-Shui-Ling, J. Bolander, L.E. Rustom, A.W. Johnson, F.P. Luyten, C. Picart, Bone regeneration strategies: Engineered scaffolds, bioactive molecules and stem cells current stage and future perspectives, Biomaterials 180 (2018) 143-162.

[5] T.A. Einhorn, L.C. Gerstenfeld, Fracture healing: mechanisms and interventions, Nat Rev Rheumatol 11(1) (2015) 45-54.

[6] A. Wubneh, E.K. Tsekoura, C. Ayranci, H. Uludag, Current state of fabrication technologies and materials for bone tissue engineering, Acta Biomater 80 (2018) 1-30.

[7] H.D. Kim, S. Amirthalingam, S.L. Kim, S.S. Lee, J. Rangasamy, N.S. Hwang, Biomimetic Materials and Fabrication Approaches for Bone Tissue Engineering, Adv Healthc Mater 6(23) (2017).

[8] A. Vishwakarma, N.S. Bhise, M.B. Evangelista, J. Rouwkema, M.R. Dokmeci, A.M. Ghaemmaghami, N.E. Vrana, A. Khademhosseini, Engineering Immunomodulatory Biomaterials To Tune the Inflammatory Response, Trends Biotechnol 34(6) (2016) 470-482.

[9] N.A. Hotaling, L. Tang, D.J. Irvine, J.E. Babensee, Biomaterial Strategies for Immunomodulation, Annu Rev Biomed Eng 17 (2015) 317-49.

[10] J.M. Anderson, A. Rodriguez, D.T. Chang, Foreign body reaction to biomaterials, Semin Immunol 20(2) (2008) 86-100.

[11] D.P. Vasconcelos, A.P. Aguas, M.A. Barbosa, P. Pelegrin, J.N. Barbosa, The inflammasome in host response to biomaterials: Bridging inflammation and tissue regeneration, Acta Biomater 83 (2019) 1-12.

[12] B. Bellich, I. D'Agostino, S. Semeraro, A. Gamini, A. Cesaro, "The Good, the Bad and the Ugly" of Chitosans, Mar Drugs 14(5) (2016).

[13] M.I. Oliveira, S.G. Santos, M.J. Oliveira, A.L. Torres, M.A. Barbosa, Chitosan drives anti-inflammatory macrophage polarisation and pro-inflammatory dendritic cell stimulation, Eur Cell Mater 24 (2012) 136-52; discussion 152-3.

[14] M.I. Oliveira, M.L. Pinto, R.M. Goncalves, M.C.L. Martins, S.G. Santos, M.A. Barbosa, Adsorbed Fibrinogen stimulates TLR-4 on monocytes and induces BMP-2 expression, Acta Biomater 49 (2017) 296-305.

[15] D.P. Vasconcelos, A.C. Fonseca, M. Costa, I.F. Amaral, M.A. Barbosa, A.P. Aguas, J.N. Barbosa, Macrophage polarization following chitosan implantation, Biomaterials 34(38) (2013) 9952-9.

[16] F. Castro, M.L. Pinto, A.M. Silva, C.L. Pereira, G.Q. Teixeira, M. Gomez-Lazaro, S.G. Santos, M.A. Barbosa, R.M. Goncalves, M.J. Oliveira, Pro-inflammatory chitosan/poly(gamma-glutamic acid) nanoparticles modulate human antigen-presenting cells phenotype and revert their pro-invasive capacity, Acta Biomater 63 (2017) 96-109.

[17] J. Tu, Y. Xu, J. Xu, Y. Ling, Y. Cai, Chitosan nanoparticles reduce LPS-induced inflammatory reaction via inhibition of NF-kappaB pathway in Caco-2 cells, Int J Biol Macromol 86 (2016) 848-56.

[18] D. Fong, M.B. Ariganello, J. Girard-Lauziere, C.D. Hoemann, Biodegradable chitosan microparticles induce delayed STAT-1 activation and lead to distinct cytokine responses in differentially polarized human macrophages in vitro, Acta Biomater 12 (2015) 183-194.

[19] D. Fong, P. Gregoire-Gelinas, A.P. Cheng, T. Mezheritsky, M. Lavertu, S. Sato, C.D. Hoemann, Lysosomal rupture induced by structurally distinct chitosans either promotes a type 1 IFN response or activates the inflammasome in macrophages, Biomaterials 129 (2017) 127-138.

[20] C.L. Bueter, C.K. Lee, V.A. Rathinam, G.J. Healy, C.H. Taron, C.A. Specht, S.M. Levitz, Chitosan but not chitin activates the inflammasome by a mechanism dependent upon phagocytosis, J Biol Chem 286(41) (2011) 35447-55.

[21] C.L. Bueter, C.K. Lee, J.P. Wang, G.R. Ostroff, C.A. Specht, S.M. Levitz, Spectrum and mechanisms of inflammasome activation by chitosan, J Immunol 192(12) (2014) 5943-51.

[22] D. Fong, C.D. Hoemann, Chitosan immunomodulatory properties: perspectives on the impact of structural properties and dosage, Future Sci OA 4(1) (2018) FSO225.

[23] K. Schroder, J. Tschopp, The inflammasomes, Cell 140(6) (2010) 821-32.

[24] M. Lamkanfi, V.M. Dixit, Mechanisms and functions of inflammasomes, Cell 157(5) (2014) 1013-22.

[25] X. Liu, Z. Zhang, J. Ruan, Y. Pan, V.G. Magupalli, H. Wu, J. Lieberman, Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores, Nature 535(7610) (2016) 153-8.

[26] M. Rayamajhi, Y. Zhang, E.A. Miao, Detection of pyroptosis by measuring released lactate dehydrogenase activity, Methods Mol Biol 1040 (2013) 85-90.

[27] E. Latz, T.S. Xiao, A. Stutz, Activation and regulation of the inflammasomes, Nat Rev Immunol 13(6) (2013) 397-411.

[28] E. Vigano, C.E. Diamond, R. Spreafico, A. Balachander, R.M. Sobota, A. Mortellaro, Human caspase-4 and caspase-5 regulate the one-step non-canonical inflammasome activation in monocytes, Nat Commun 6 (2015) 8761.

[29] M. Monteleone, A.C. Stanley, K.W. Chen, D.L. Brown, J.S. Bezbradica, J.B. von Pein, C.L. Holley, D. Boucher, M.R. Shakespear, R. Kapetanovic, V. Rolfes, M.J. Sweet, J.L. Stow, K. Schroder, Interleukin-1beta Maturation Triggers Its Relocation to the Plasma Membrane for Gasdermin-D-Dependent and -Independent Secretion, Cell Rep 24(6) (2018) 1425-1433.

[30] C.L. Evavold, J. Ruan, Y. Tan, S. Xia, H. Wu, J.C. Kagan, The Pore-Forming Protein Gasdermin D Regulates Interleukin-1 Secretion from Living Macrophages, Immunity 48(1) (2018) 35-44 e6.

[31] Z. Julier, A.J. Park, P.S. Briquez, M.M. Martino, Promoting tissue regeneration by modulating the immune system, Acta Biomater 53 (2017) 13-28.

[32] C.A. Sridharan R., Kelly DJ., Kearney CJ., O'Brien FJ, Biomaterial based modulation of macrophage polarization: a review and suggested design principles, Materials today 18(6) (2015) 313-325.

[33] D. Hachim, S.T. LoPresti, C.C. Yates, B.N. Brown, Shifts in macrophage phenotype at the biomaterial interface via IL-4 eluting coatings are associated with improved implant integration, Biomaterials 112 (2017) 95-107.

[34] K.L. Spiller, S. Nassiri, C.E. Witherel, R.R. Anfang, J. Ng, K.R. Nakazawa, T. Yu, G. Vunjak-Novakovic, Sequential delivery of immunomodulatory cytokines to facilitate the M1-to-M2 transition of macrophages and enhance vascularization of bone scaffolds, Biomaterials 37 (2015) 194-207.

[35] T. Li, M. Peng, Z. Yang, X. Zhou, Y. Deng, C. Jiang, M. Xiao, J. Wang, 3D-printed IFN-gamma-loading calcium silicate-beta-tricalcium phosphate scaffold sequentially activates M1 and M2 polarization of macrophages to promote vascularization of tissue engineering bone, Acta Biomater 71 (2018) 96-107.

[36] J.R. Alhamdi, T. Peng, I.M. Al-Naggar, K.L. Hawley, K.L. Spiller, L.T. Kuhn, Controlled M1-to-M2 transition of aged macrophages by calcium phosphate coatings, Biomaterials (2018).

[37] D.P. Vasconcelos, M. Costa, I.F. Amaral, M.A. Barbosa, A.P. Aguas, J.N. Barbosa, Modulation of the inflammatory response to chitosan through M2 macrophage polarization using pro-resolution mediators, Biomaterials 37 (2015) 116-23.

[38] C. Di Tommaso, C. Como, R. Gurny, M. Moller, Investigations on the lyophilisation of MPEG-hexPLA micelle based pharmaceutical formulations, Eur J Pharm Sci 40(1) (2010) 38-47.

[39] M.D. Howard, X. Lu, M. Jay, T.D. Dziubla, Optimization of the lyophilization process for long-term stability of solid-lipid nanoparticles, Drug Dev Ind Pharm 38(10) (2012) 1270-9.

[40] Y.P. Sun, S.F. Oh, J. Uddin, R. Yang, K. Gotlinger, E. Campbell, S.P. Colgan, N.A. Petasis, C.N. Serhan, Resolvin D1 and its aspirin-triggered 17R epimer. Stereochemical assignments, anti-inflammatory properties, and enzymatic inactivation, J Biol Chem 282(13) (2007) 9323-34.

[41] B.N. Brown, R. Londono, S. Tottey, L. Zhang, K.A. Kukla, M.T. Wolf, K.A. Daly, J.E. Reing, S.F. Badylak, Macrophage phenotype as a predictor of constructive remodeling following the implantation of biologically derived surgical mesh materials, Acta Biomater 8(3) (2012) 978-87.

[42] L. Xing, E.M. Schwarz, B.F. Boyce, Osteoclast precursors, RANKL/RANK, and immunology, Immunol Rev 208 (2005) 19-29.

[43] L. Batoon, S.M. Millard, M.E. Wullschleger, C. Preda, A.C. Wu, S. Kaur, H.W. Tseng, D.A. Hume, J.P. Levesque, L.J. Raggatt, A.R. Pettit, CD169(+) macrophages are critical for osteoblast maintenance and promote intramembranous and endochondral ossification during bone repair, Biomaterials (2017).

[44] C. Schlundt, T. El Khassawna, A. Serra, A. Dienelt, S. Wendler, H. Schell, N. van Rooijen, A. Radbruch, R. Lucius, S. Hartmann, G.N. Duda, K. Schmidt-Bleek, Macrophages in bone fracture healing: Their essential role in endochondral ossification, Bone 106 (2018) 78-89.

[45] J. Pajarinen, T. Lin, E. Gibon, Y. Kohno, M. Maruyama, K. Nathan, L. Lu, Z. Yao, S.B. Goodman, Mesenchymal stem cell-macrophage crosstalk and bone healing, Biomaterials (2018).

[46] A.C. Wu, L.J. Raggatt, K.A. Alexander, A.R. Pettit, Unraveling macrophage contributions to bone repair, Bonekey Rep 2 (2013) 373.

[47] A. Schindeler, M.M. McDonald, P. Bokko, D.G. Little, Bone remodeling during fracture repair: The cellular picture, Semin Cell Dev Biol 19(5) (2008) 459-66.

[48] L. Vi, G.S. Baht, H. Whetstone, A. Ng, Q. Wei, R. Poon, S. Mylvaganam, M. Grynpas, B.A. Alman, Macrophages promote osteoblastic differentiation in-vivo: implications in fracture repair and bone homeostasis, J Bone Miner Res 30(6) (2015) 1090-102.

[49] T.A. Wynn, K.M. Vannella, Macrophages in Tissue Repair, Regeneration, and Fibrosis, Immunity 44(3) (2016) 450-462.

[50] S. Viguet-Carrin, P. Garnero, P.D. Delmas, The role of collagen in bone strength, Osteoporos Int 17(3) (2006) 319-36.

[51] L. Junqueira, Bignolas, G., Brentani, RR. , Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections, Histochem J 11(4) (1979) 447-455.
[52] M. Narvekar, H.Y. Xue, J.Y. Eoh, H.L. Wong, Nanocarrier for poorly water-soluble anticancer drugs--barriers of translation and solutions, AAPS PharmSciTech 15(4) (2014) 822-33.
[53] A.A. Attama, C.E. Umeyor, The use of solid lipid nanoparticles for sustained drug release, Ther Deliv 6(6) (2015) 669-8